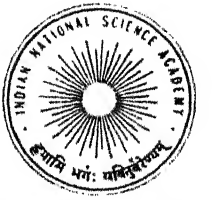
Indo-Soviet Symposium on the Chemistry of Natural Products

PUNE
January 28–February 1
1981

Plenary Lectures



INDIAN NATIONAL SCIENCE ACADEMY
NEW DELHI-110002

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PREFACE

INDO-SOVIET SYMPOSIA on the Chemistry of Natural Products have been held regularly since the holding of the First Symposium in Tashkent from September 17–21, 1968. The credit for initiating this programme goes to Professor T. R. Seshadri and Professor M. M. Shemyakin who visualised the importance of the subject and took deep personal interest in organising the first two symposia. Even after the sad demise of these stalwarts, the two Academies, Indian National Science Academy and USSR Academy of Sciences, have been organising these Symposia, on a regular basis, alternatively in USSR and in India. So far six Symposia have been held, thrice in USSR (Tashkent—September, 1968; Tashkent—October, 1973; Yeravan—May, 1978) and thrice in India (New Delhi—February, 1970; Lucknow—February 1976; Pune—January, 1981). On the whole, each one of these Symposia proved a success in that some excellent presentations were made and also it gave an opportunity to scientists on both sides to know each other at a personal level, and thus help build mutual understanding and encourage scientific exchange.

Sixth Indo-Soviet Symposium on the Chemistry of Natural Products was held from January 28 - February 1, 1981 at National Chemical Laboratory, Pune. Some 13 Scientists from USSR participated. The number of Indian participants was around 120. In all, ten plenary lectures were delivered and many invited half-hour lectures were presented at two concurrent sessions. It was felt that it would be worthwhile to get the plenary lectures published by the Academy. It is in this context that the present supplement has been organised. Out of the ten plenary lectures, only 9 are being published, since Professor S. C. Bhattacharyya who delivered the tenth plenary lecture has been unable to prepare the manuscript because of several local conditions and suggested that we may go ahead with the publication, as scheduled. The plenary lectures presented at an Indo-Soviet Symposium on the Chemistry of Natural Products are being published for the first time and I do hope that this practice would be followed in future as well.

SUKH DEV
Malti-Chem Research Centre
Nandesari, Baroda

August 13, 1981

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Bioorganic Chemistry

METHODS FOR PREPARATION OF PHYSIOLOGICALLY ACTIVE POLYOXYGENATED CHOLESTANE DERIVATIVES

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(Received 19 October 1981)

The authors have elaborated some improved methods of introducing oxygen functions (or additional double bond) into natural sterols. Such an elaboration has led to the proposal of new procedures for the preparation of vitamin D and its 3-fluoro-analogue. By chromic acid or potassium permanganate oxidation of ergosterol and some further transformations, series of 5-, 6-, 7- and 14-oxygenated compounds have been obtained, among them 2-deoxypoststerone. On the basis of C_{20}- and C_{21}-intermediates containing 20-keto- or 22-aldehyde groups the synthesis of 2-desoxyecdysone and 2-desoxyecdysterone have been accomplished. They possess a high moulting hormonal activity. To build the oxygenated side chain the authors have used a Grignard reaction with isopentenylmagnesium chloride, oxidation with mercury acetate, performic acid and other agents.

Starting from pregnenolone or dehydro-epi-androsterone acetates some polyhydroxy-cholesterols have been prepared which can be transformed into 25-hydroxyprecalciferol and some other derivatives important from the medical view point.

Having used a Favorski condensation of diacetylene with pregnenolone acetate it became possible after subsequent transformation to come to 20-hydroxyabridin.

Keywords: Ergosterol; Cholesterol Analogs; Sterol Side Chain; Ecdysones; Brassinolide.

INTRODUCTION

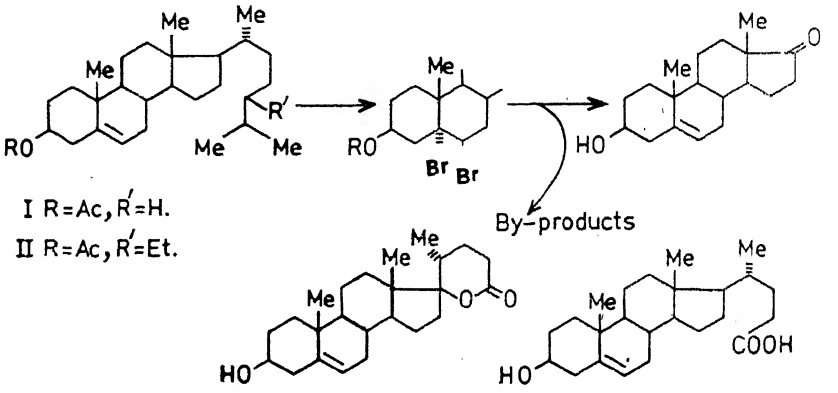
LOOKING at the steroid biosynthesis in plant and animal organisms one can see how gradually from primary compounds—cholesterol and lanosterol—by oxidative processes the physiologically active compounds are formed. These active and hormonal compounds include corticoids, cardiac aglycones, moulting insect hormones,complicated alkaloids and so on (Heftmann, 1970). It is clear that just the presence of oxygen functions (hydroxy-, keto- and carboxyl groups) determines their importance and regulating abilities in organisms.

A partial synthesis of these polyfunctional steroids is a big problem in steroid chemistry and this problem is more difficult, when there are less number of functional groups (hydroxy groups and double bonds) in the structure of the primary compound.

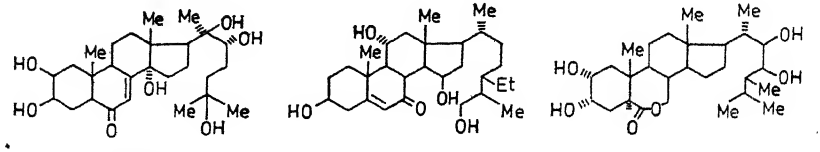
Ruzicka succeeded in converting cholestanol-3α acetate to androsterone acetate (Ruzicka *et al.*, 1934) by chromic acid oxidation only in a negligible yield 0.2 per cent.

In the forties, the pharmaceutical industry used for a short period the oxidation

of available cholesterol acetate (I) into dehydroepiandrosterone (with protection of the double bond by bromination) with a yield (according to patent data) amounting to 18–20 per cent. Some side-products (lactones, acids) were also formed. However, the application of chemical methods to such a cheap substance as sitosterol acetate (II) failed (the yield did not exceed 8–9 per cent) and only a recent discovery by Vovchka (1977) in the USA (Upjohn Co) a microbiological degradation of sitosterol made it an important starting material for the partial synthesis of steroid hormones.



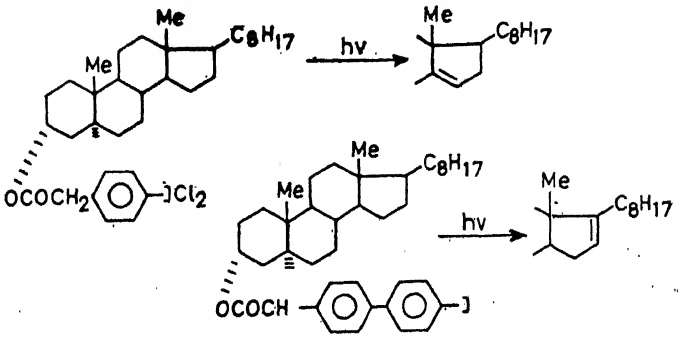
Besides hormones with a shortened side chain to which belong corticoids, cardiac aglycones and several alkaloids there are physiologically active compounds with a full side chain carrying some oxygen functions. Such are for instance ecdysones (mentioned above), provitamins of the group D (25-hydroxy- and 24,25-dihydroxycholesterols), a sex hormone of algae oogoniol, a growth promoting steroid brassinolide from the pollen of raps and so on.



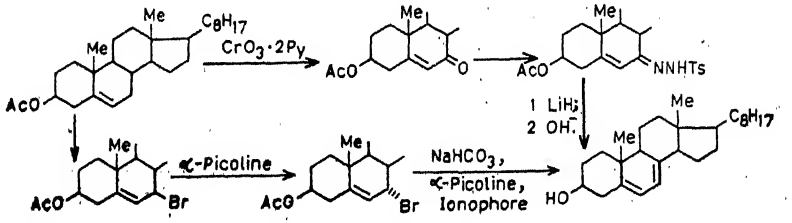
Ecdysterone Oogoniol Brassinolide

The synthesis of these and analogous compounds has its special peculiarities and difficulties. I shall not make a survey of different approaches to this problem because it would take much time and I allow myself to fix on the interesting method of "animation" of the sterol molecule proposed by Professor R. Breslow (Columbia University, New York). He successfully used a photoreaction of 3-acyloxyderivatives

with relatively long acyl groups containing a iodosubstituted aromatic ring at the end of the chain (Breslow *et al.*, 1974).

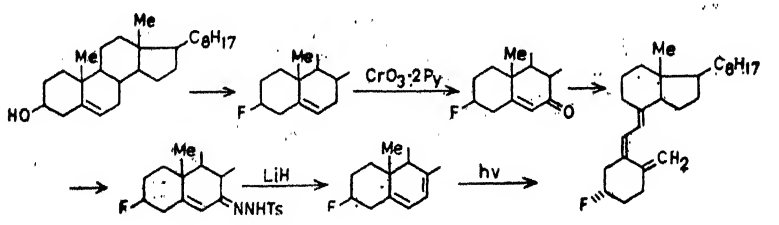


We focused our attention on oxidative transformation at the ring B of steroids with the 5, 6-double bond. It is well known that by allylic bromination and dehydrobromination it is possible to introduce a second double bond in the ring B at the position 7, 8. That conjugated dienic system exists in some sterols, for instance, in ergosterol. We succeeded in improving the method of preparation of $\Delta^{5,7}$-dienes *via* Δ^{5}-7-bromides and proposed a new method to obtain the dienes through Δ^{5}-7-ketones (Yablonskaya & Segal, 1973).



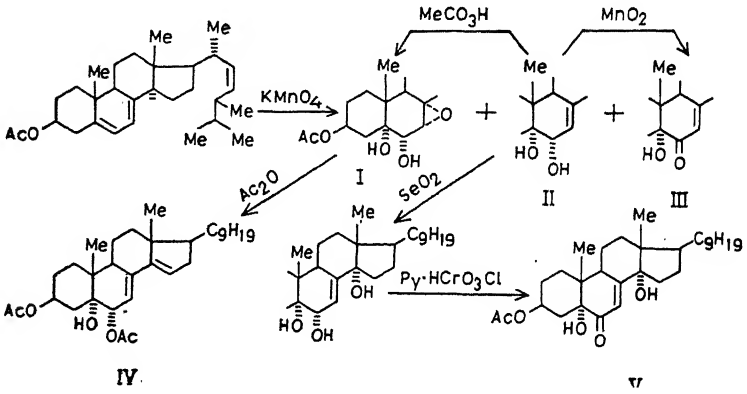
An improvement of the yields in the first method was achieved, first by using of α-picoline, which converts (at 20°) the 7β-bromo-derivative into more reactive 7 α-isomer and, secondly, by adding an ionophore at the dehydrobromination step that affords only the $\Delta^{5,7}$-diene.

The second method is also efficient and can be used not only in the cholestane but also in androstane series, 3-fluorocholecalciferol was just obtained by this method (Yakhimovich *et al.*, 1976).

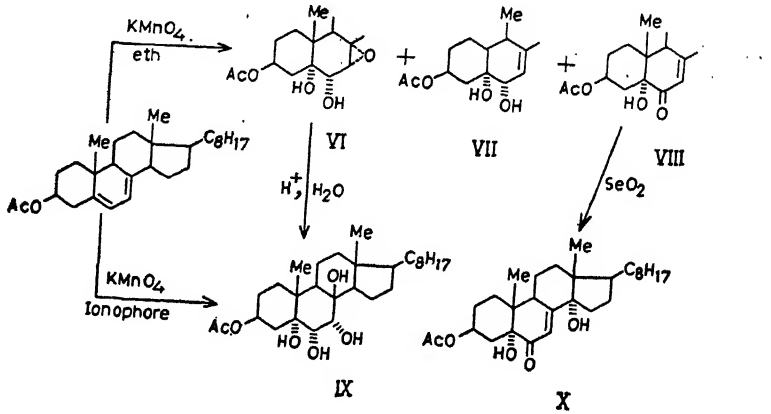


Further, we investigated oxidation at $\Delta^{5,7}$-steroid dienes. At one time the oxidation of ergosterol and cholestrol were the subject of many works by Windaus, Burawoy, Fieser and other chemists. We were interested in this problem from the point of view of the possibility to obtain products for the synthesis of ecdysone-like compounds.

The oxidation of ergosterol acetate by aqueous potassium permanganate in cyclohexane at 80° turned out to be most interesting (Segal & Torgov, 1981 *a*, *b*). After chromatography on silica gel, we succeeded in isolating three compounds (I-III) in yields 25, 6 and 10 per cent respectively.



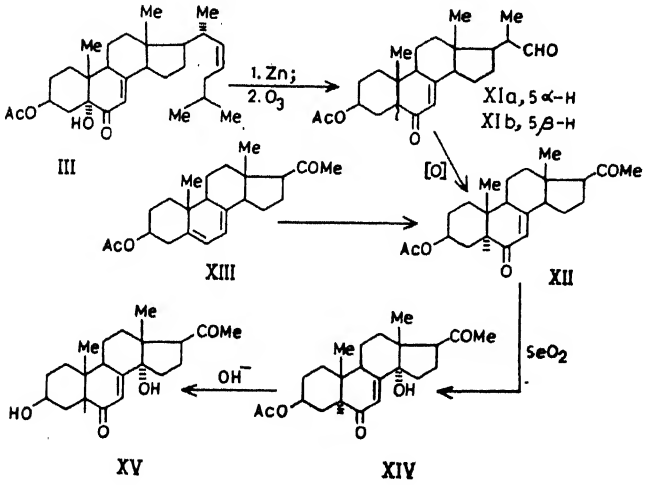
The compound (III) proved to be identical with Burawoy ketone which was further used by us for the synthesis. By MnO_2 oxidation the compound (II) gives rise to Burawoy ketone (III) and by treating with paracetic acid affords the main product (I). Acetylation of I leads to "Fieser product" (IV). By subsequent oxidation with selenium oxide and pyridinium chlorochromate (other oxidative agents are not useful) II was converted into trihydroxyketone (V) with the grouping typical for ecdysones. Under other conditions, namely by chromic acid oxidation in ether solution it proved possible to raise the yield of Burawoy ketone up to 70 per cent (Yablonskaya & Segal, 1971) and make it available. Oxidation of 7-dehydrocholesterol by aqueous



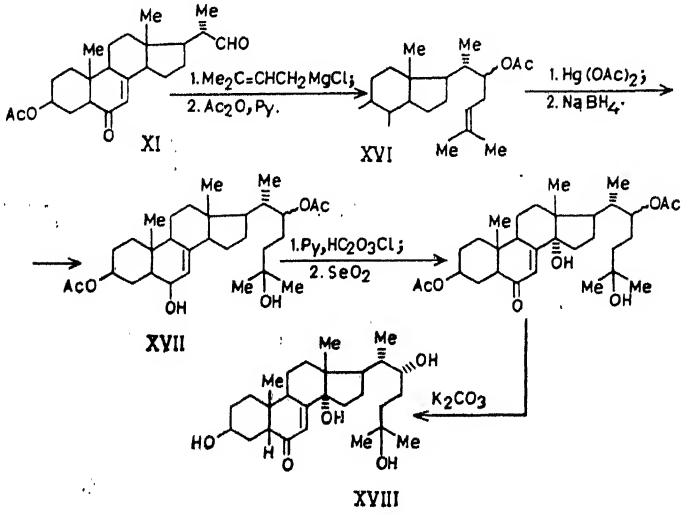
potassium permanganate solution in cyclohexane gave results qualitatively analogous to those for oxidation of ergosterol. Three compounds (VI-VIII) were isolated in yields 71, 7 and 2 per cent respectively. By oxidation of dehydrocholesterol with permanganate in the presence of an ionophore the pentaol acetate (IX) was obtained (in a 50 per cent yield).

A mild hydrolysis of the epoxide (VI) gives the pentaol acetate (IX). Selenium oxide oxidation of (VIII) affords the compound (X), an analogue of V.

The above mentioned Burawoy ketone (II) was converted into 2-desoxypoststerone by a series of the following transformations:



The 5α-hydroxy group was removed by zinc reduction and the mixture of the 5α- and 5β-compounds was subjected to ozonolysis giving rise to a mixture of the aldehydes (XI*a* and XI*b*). Oxidation of this mixture (by air in the presence of tertiary butoxide) gives pregn-7-endione-6, 20-ol-3 β -acetate (XII) which was also prepared

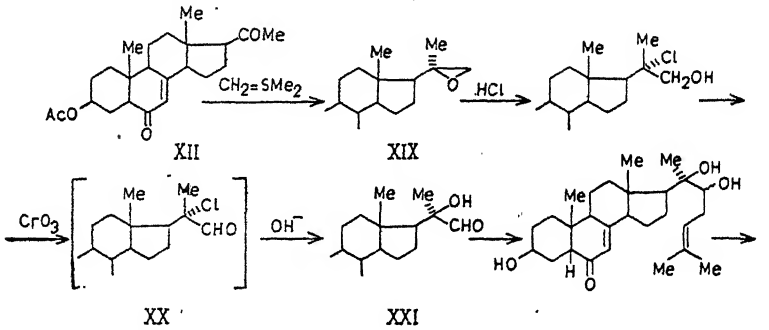


from the corresponding $\Delta^{5,7}$-derivative (XIII). Selenium oxide oxidation and subsequent alkaline hydrolysis accompanying with the inversion at C_5 leads to 2-desoxypoststerone (XV).

The compunds XI and XII appeared to be starting materials for the synthesis of 2-desoxyecdysone and 2-desoxyecdysterone (cf. Segal & Torgov, 1979).

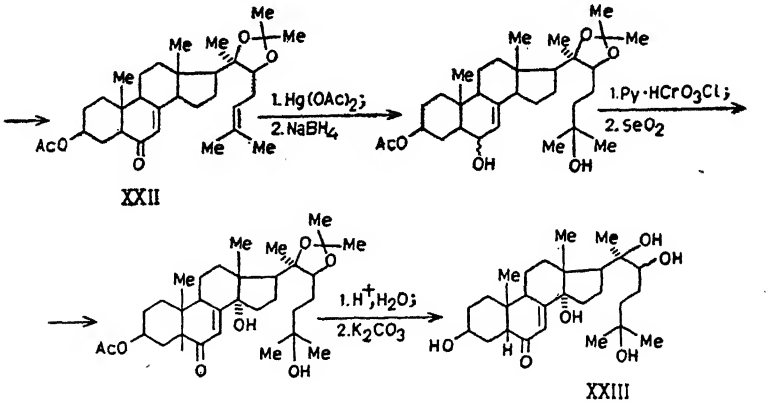
Grignard reaction of the aldehyde (XI) with isopentenylmagnesium chloride at −40° led (after acetylation) to the cholestane derivative (XVI). Mercury acetate addition to the latter and subsequent reduction gave the tetrahydroxycompound acetate (XVIII). The selective oxidation of the compound (XVIII) by pyridinium chlorochromate (oxidation of 6-OH group) and then by selenium oxide (introduction of a hydroxy group in the allylic position 14) gives rise to the acetate and after saponification (and epimerisation at C_5) to 2-desoxyecdysone (XVIII) with the small amounts of its isomer.

A similar route was chosen for synthesis of 2-desoxyecdysterone (XXIII) differing from 2-desoxyecdysone by the additional 20-hydroxy group.



XII XIX XX XXI

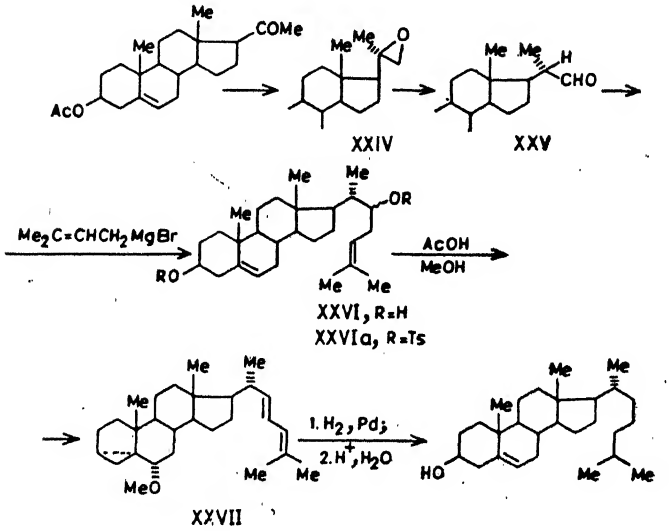
The starting material was the pregnane derivative (XII) which after the reaction with methylene dimethylsulphide gave the oxirane (XIX). The splitting of the oxirane ring by hydrogen chloride, the subsequent oxidation to the aldehyde (XX) and the alkaline hydrolysis lead (with the inversion at C_{20}) to C_{22}-hydroxyaldehyde of the desirable ("natural") configuration. The further steps were practically the same as in the case of 2-desoxyecdysone.



XXII XXIII

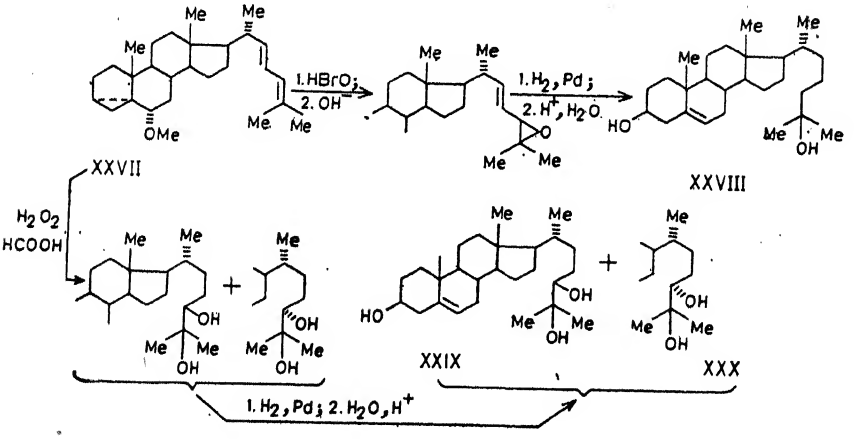
The only particular detail of the last steps was an acetonide protection.

The method of constructing the cholestane side chain by the reaction of 20-keto-pregnanes with isopentenylmagnesium chloride was applied by us also for preparation of cholesterol and a number of its derivatives with hydroxy groups in positions 22, 24 and 25 (Segal & Torgov, 1979)



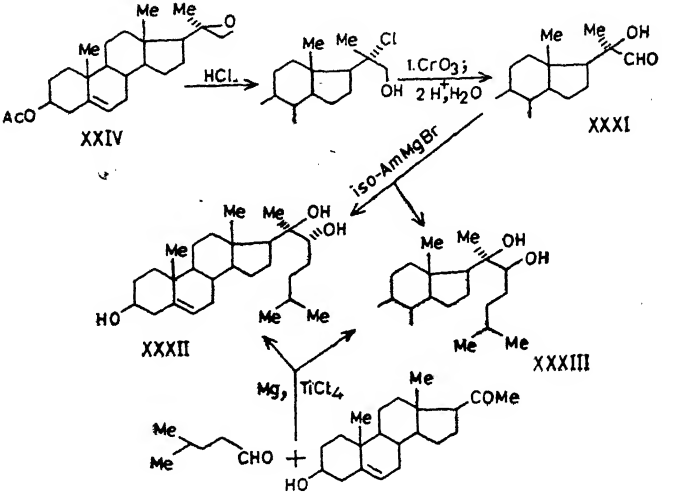
Starting from pregnenolone acetate *via* the oxide (XXIV) the aldehyde (XXV) was obtained which was converted by Grignard reaction to the cholestadienol (XXVI). Treatment of its ditosylate (XXVI*a*) with potassium acetate in methanol gave the dienic isosteroid (XXVII), hydrogenation of which with subsequent hydrolysis afforded cholesterol. By this series of reactions the stereochemistry of the epoxide (XXIV) and the course of its fission was confirmed.

Addition of the elements of hypobromous acid to the dienic system of the i-steroid (XXVII) and the subsequent transformations according to the following scheme led to the 25-hydroxycholesterol (XXVIII).



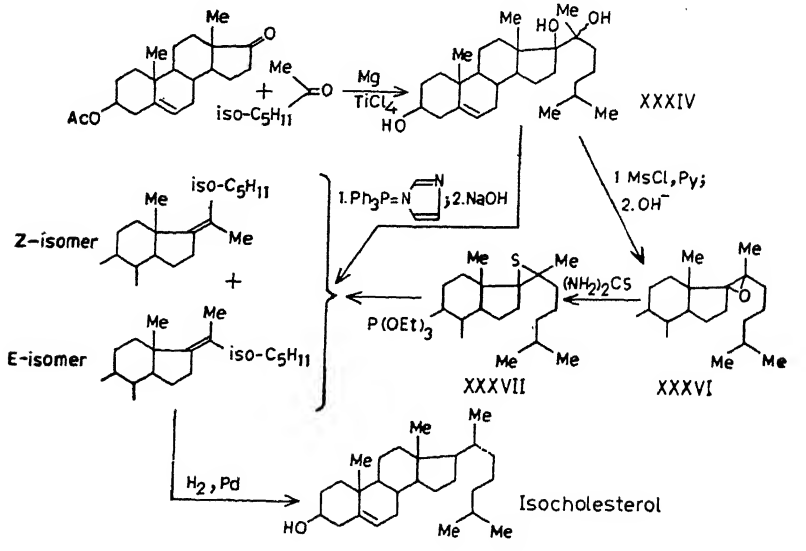
Oxidation of XXVII with performic acid, hydrogenation and hydrolysis gave rise to the isomeric 24R, 25-dihydroxy and 24S, 25-dihydroxycholesterols (XXIX and XXX).

Starting from the intermediate epoxide (XXIV) *via* the aldehyde (XXXI) the 20R, 22R-dihydroxy and 20R, 22S-dihydroxycholesterols (XXXII) and (XXXIII) were obtained (Sydkov & Segal, 1977).



Both dihydroxycholesterols can be obtained in a much simpler way starting from available pregnenolone and 4-methylpentanol by reductive condensation with magnesium in the presence of titanium tetrachloride. Herewith both isomers are formed approximately in equal amounts in a yield of 80 per cent.

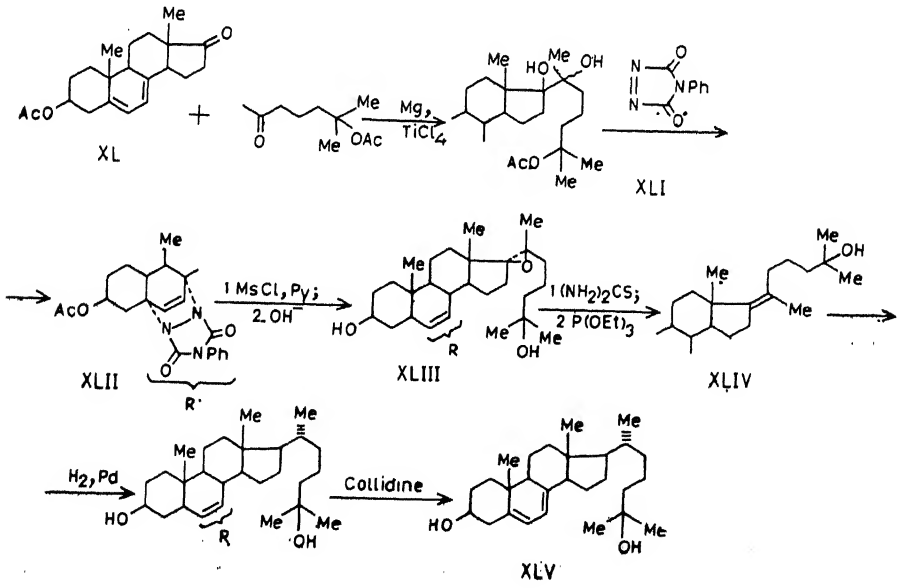
We made use of this reaction of reductive condensation in some other cases. It



turned out to be that it can be applied not only for 20-ketopregnanes with the active keto-group but for 17-ketosteroids as well, where the keto-group is less active. So, starting from dehydroepiandrosterone acetate and 7-methylheptanone-2 (both products are available on industrial scale) a mixture of 17, 20-dihydroxycholesterols (XXXIV) can be obtained (Segal & Torgov, 1981 *a,b*).

After treatment of the mixture XXXIV with triphenylimidazolyl-phosphonium salt a mixture of the Z- and E-isomers of 17 (20)-dehydrocholesterols (XXXV) in ratio 1 : 7 is formed. Another route of the transformation of the dihydroxycholesterols (XXXIV) *via* the epoxide (XXXVI) and the thiirane (XXXVII) gives the mixture of the Z- and E-compounds in ratio 8:1. Hydrogenation of the Z-isomer leads to cholesterol, hydrogenation of the E-isomer to 20-isocholesterol.

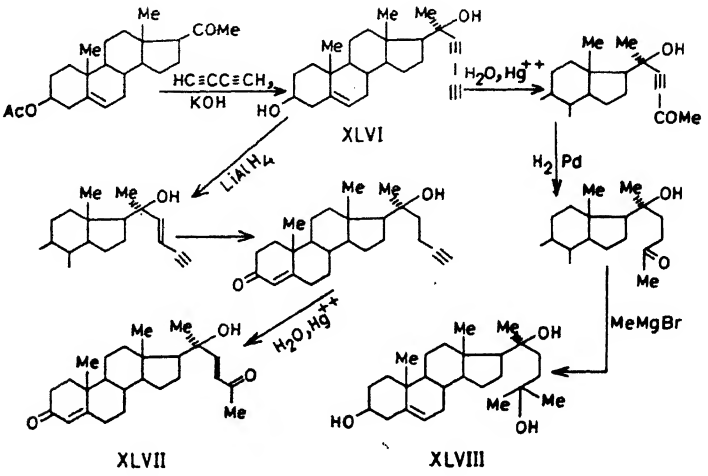
Among oxygenated cholesterols the synthesis of which we demonstrated above the 25-hydroxycholesterol (XXVII) and 24R, 25-dihydroxycholesterol (XXIX) are of great interest. These compounds can be used as intermediates in the synthesis of the 25-hydroxycholecalciferol (XXXVIII) and the 24R, 25-dihydroxycholecalciferol (XXXIX)—the potent antirachitic preparations, much more active than the traditional vitamin D. Certainly, the route to antirachitic vitamins can include first the creation of the $\Delta^{5,7}$-dienic system and only afterwards the creation or transformation of the side cholestane chain. One of the variants can be seen on the following scheme:



Reductive condensation of the bisdehydroandrosterone acetate (XL) with 6-methyl-6-acetoxyheptanone-2 under the action of magnesium proceeded quite satisfactorily giving rise to a mixture of polyhydroxycholestadienes (XLI). To carry out further transformations it was necessary to have the reactive dienic system protected. For this purpose the phenylimide of azodicarbonic acid suits best of all. Treatment

of the adduct (XLII) with mesylchloride and then with alkali led to the epoxide (XLIII). Transformation of the latter to the thiirane and splitting off the sulphur atom have been described above. The subsequent selective hydrogenation of the compound (XLIV) and elimination of the protecting group by heating with collidine gave rise to 25-hydroxyprecalciferol (XLV).

In conclusion, we would like to draw your attention on the way of creating other than cholestane oxygenated side chains in the steroid molecule. One of these ways includes introducing the diacetylenic radical in the position 20 of pregnenolone in order to utilize a high reactivity of the triple bonds.



By combination of reactions of reduction and hydration of the triple bond (according to Kutcherov) of the carbinol (XLVI) we obtained 20-hydroxyabridin (XLVII) and 20R, 25-dihydroxy-24-norcholesterol (XLVIII). Abridin itself isolated from seeds of *Abrus precatorius* Linn possesses a contraceptive action.

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Biomembrane

AN APPROACH TO TISSUE TARGETING OF DRUGS AND PROTEINS USING LIPOSOMES

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(*Received 7 September 1981*)

Grafting of different glycosides on the surface of liposomes followed by administration through tail vein in rats indicates that galactosylated and mannosylated liposomes are more efficient in transporting liposome-entrapped material to liver. Further studies on the uptake of liposomes by isolated cell types of rat liver after *in vivo* administration reveal that hepatocytes are three times more efficient than non-parenchymal cells in taking up liposomes having β-galactoside on their surface whereas α-mannoside-liposomes are taken up preferentially by non-parenchymal cells. Non-sugar liposomes are taken up by both cells. Glycoside containing liposomes are also cleared from the circulation at a faster rate than non-sugar liposomes. Asialofetuin and mannan inhibit both the clearance and the uptake by isolated cells of β-gal- and α-man-liposomes, respectively. These findings show that surface β-galactoside and α-mannoside can mediate selective targeting of liposomes towards parenchymal and non-parenchymal cells respectively of rat liver. Considering the efficacy of glycosylated liposomes in transporting entrapped material towards specific cell types of liver, studies were made with uridine entrapped in different glycosylated liposomes to evaluate their capacity in regenerating D-galactosamine-induced hepatitis in rats in which parenchymal cells are mainly affected. It was observed that galactosylated liposome-entrapped uridine was more efficient in regenerating the hepatitis liver as compared to other liposomes studied.

Keywords: Glycosylated Liposomes; γ-globulin; Tissue Targeting; D-Galactosamine-induced Hepatitis

INTRODUCTION

IN recent years liposomes have gained wide acceptance as carriers of biologically important molecules (Tyrrell *et al.*, 1976; Papahadjapoulos, 1978; Ghosh & Bachhawat, 1980 *a*, *b*; and Gregoriadis, 1980). Since the targeting of liposomes towards specific tissues is severely limited by the rapid sequestration of liposomes by the reticulo-endothelial system, attempts have been made to prepare functional liposomes for effective targeting utilizing specific ligand-receptor interactions. Earlier work from this laboratory has opened up potential application of glycolipids in their use as ligands for specific targeting of liposomes as their orientation on the liposomal surface allows the terminal sugar residues to interact with lectin having the binding properties for that particular glycoside residue (Surolia *et al.*, 1975). Furthermore, the kinetic studies revealed that this particular interaction was dependent on several

factors, such as the density of glycoside residues on the surface of liposomes, the chain length of oligosaccharides incorporated into the liposomes and also the phase transition temperature of the phospholipid used for making liposomes (Surolia *et al.*, 1975; and Surolia & Bachhawat, 1978 *a, b*). These facts and the information available regarding the presence of hepatic lectin on the surface of parenchymal cells by the brilliant work of Ashwell and Morell and their coworkers led us to design the *in vivo* experiment with the help of GM_1 ganglioside where we had incorporated the ganglioside in such a way that the oligosaccharide portion of the glycolipid was at a high density on the surface of liposomes (Surolia & Bachhawat, 1977). When invertase entrapped in such liposomes was administered intravenously to rats, a rapid uptake of the enzyme into the liver was observed within 15m, after administration. Subcellular location of most of the enzyme taken up by the liver in lysosomal fraction suggested that the uptake was primarily through the endocytotic process. That this endocytotic process was mediated by galactose receptor had further been confirmed by competitive inhibition of the uptake by asialofetuin having galactose as the terminal sugar residue. However, a number of investigators were unable to confirm this observation and had suggested that the terminal glycoside residue on the liposome may not have any particular specificity (Grigoriadis & Neerunjun, 1974; and Jonah *et al.*, 1978). This led us to reinvestigate further the role of different glycoside residues on the surface of liposomes and their uptake by various organs. A number of different glycosides such as β-galactoside, α-galactoside, α-mannoside and β-N-acetylglucosaminide were grafted on the surface of liposomes and it was observed that enhanced liposome uptake by liver could be achieved by grafting galactoside and mannoside on the surface of liposomes (Ghosh & Bachhawat, 1980 *a, b*). It may be mentioned that the anomeric form of the terminal sugar plays an important role in the *in vivo* uptake of liposomes since in our earlier studies it was shown that liposomes having β-D-galactopyranosyl residues on their surface are rapidly taken up by hepatic cells in comparison to those having α-D-galactopyranosyl residues on the surface (Ghosh *et al.*, 1981). The density-dependent uptake studies revealed an interesting fact that the density of the glycoside residues on the surface of the liposomes is the determining factor for the uptake of glycosylated liposomes by the liver (Ghosh & Bachhawat, 1980 *a, b*). When the different densities of glycosides were incorporated on the surface of liposomes, it was observed that the preferential uptake of these glycosylated liposomes by the liver was dependent on the density of glycosides. At a lower density of glycosides, there was no potential uptake by the liver. Moreover, as observed in *in vitro* studies (Surolia & Bachhawat, 1978 *a, b*), the chain length of the oligosaccharides of glycolipids has an important role in the uptake of liposomes. There was a low uptake of galactocerebroside liposomes in comparison to asialoganglioside liposomes and β-galactose liposomes (in spite of having the requisite galactose residue) which might be attributed to restricted access of the hepatocyte receptor towards the galactose residue of cerebrosides (Ghosh & Bachhawat, 1980 *a, b*). These observations led us to conclude that other investigators had not taken into consideration the critical receptor density on the surface of liposomes in designing their experiments and as such primarily the low density of the surface sugar on liposome may be the reason for their inability to confirm our previous results.

Considering the fact that the parenchymal cells contain galactose binding lectin

whereas non-parenchymal cells contain mannose binding lectin, it was thought worthwhile to explore the possibility of effective targeting of liposomes towards different cell types of rat liver by changing the nature of glycosides on the surface of liposomes. For this purpose we have used liposomally entrapped ^{125}I-γ-globulin as model biologically active macromolecule and in addition, liposomal surface has been grafted with β-galactoside and α-mannoside according to the procedure described earlier (Ghosh & Bachhawat, 1980*a*, *b*). Different glycoside grafted liposomes containing ^{125}I-γ-globulin were injected intravenously (2.5–3.5 mg lipids per rate) into rats of body weight 120–135g and the clearance of radioactivity from the circulation at different time intervals (5–60 min) as well as the distribution of ^{125}I-γ-globulin in the isolated cell types of perfused liver were examined after 15 min of administration. The amount of ^{125}I labelled-γ-globulin varied from 6–7 × 10^4 cpm for clearance

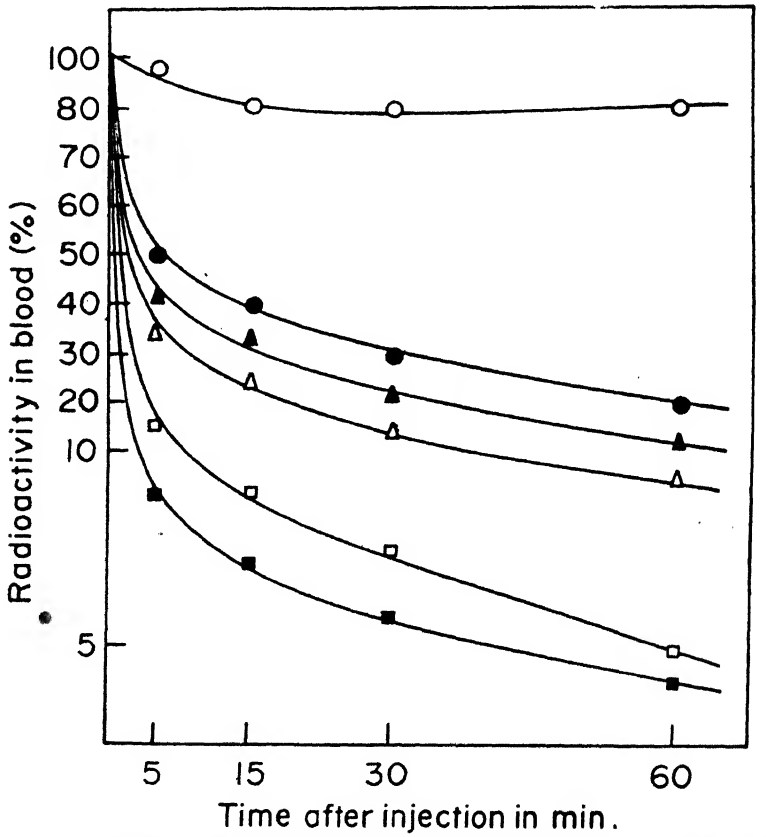

FIG. 1. Clearance of different types of liposome from the circulation of rats. Rats were injected with liposome containing ^{125}I-γ-globulin (6–7×10^5 cpm). Radioactivity was measured in the blood, the volume of which in all experiments described here was taken as 6.0 ml per 100 g body wt. and it was expressed as a percentage of the injected radioactivity per total volume of blood. Each point is the average value obtained from three rats. As a control, free γ-globulin (6×10^4 cpm) was also injected. —○——○— Free γ-globulin, —●——●— PE-liposomes; —□——□— α-man-liposome; —▲——▲— α-man-liposome+mannan; —■——■— Asialoganglioside liposome; —△——△— Asialoganglioside+asialofetuin.

studies and from 1.5–2 × 10^5 cpm for liver uptake studies. As a control, free ^{125}I-γ-globulin (6 × 10^4 cpm) was also injected. In the competition studies, 10mg of asialofetuin or mannan were administered intravenously before injection of liposomes. Parenchymal and non-parenchymal cell suspensions were obtained by the perfusion of liver *in situ* according to the method of Berry & Friend (1969) with a slight modification. Nonparenchymal cell suspensions were made free of traces of parenchymal cells by pronase treatment after the method of Mills and Zucker-Franklin (Mills & Zucker-Franklin, 1969). Cells were counted by hemocytometer.

The rates of clearance of different liposomes as monitored by entrapped ^{125}I-γ-globulin are given in Fig. 1 which shows biphasic nature. This is not surprising considering the size heterogeneity of the mixed population of multilamellar liposomes injected. Liposomes containing β-galactoside and α-mannoside were found to be cleared from the circulation at a much faster rate than those having no glycosides. The inhibition of clearance, as found in the case of β-galactoside and α-mannoside-liposome by asialofetuin and mannan respectively, is similar to that observed for galactose terminated and mannose-terminated glycoproteins (Ashwell & Morell, 1974; and Stahl & Schlesinger, 1980). The extent of uptake by the liver of ^{125}I-γ-globulin was more in the case of glycosylated liposomes indicating thereby the involvement of a specific interaction of these sugar containing liposomes with the liver. Furthermore, most of the radioactivity associated with glycosylated liposomes, which had disappeared from the circulation after 5 min. could be accounted in the liver and thereafter there was a gradual decrease in the amount of radioactivity. That liposomes are actually taken up inside the cells of liver has been shown by our earlier subcellular distribution studies which revealed the presence of most of the liposomal radioactivity in mitochondrial-lysosomal fraction. The uptake of liposome-entrapped radioactive γ-globulin by isolated cell types of rat liver after *in vivo* administration is given in Table I. Radioactive protein entrapped in nonglycosylated liposomes were taken up by both parenchymal and non-parenchymal cells, but the uptake is slightly higher in the case of non-parenchymal cells. The uptake of ^{125}I-γ-globulin by

TABLE I

Uptake of liposome-entrapped ^{125}I-labelled γ-globulin by different cell types of rat liver

Experiments	^{125}I-labelled γ-globulin injected (cpm × 10^{-5})	Radioactivity (cpm × 10^{-3}) per 5×10^6 cells ± S.D.	
		Parenchymal	Non-parenchymal
Neutral liposome	1.5	4.5±0.7	6.7±0.9
Asialoganglioside liposome	2.0	19.0±2.0	6.9±1.0
Asialoganglioside liposome + Asialofetuin	2.0	5.2±0.6	9.3±0.8
Asialoganglioside liposome + mannan	2.0	17.1±1.5	7.5±1.1
α-Man-liposome	1.5	2.0±0.5	15.0±1.3
α-Man-liposome + Mannan	1.5	4.4±0.6	5.2±1.0
α-Man-liposome + Asialofetuin	1.5	2.6±0.5	13.8±1.1

Rats were injected with different liposomes containing ^{125}I-labelled γ-globulin with or without 8–10 mg asialofetuin and mannan and after 15 min liver cell suspension were obtained by the perfusion of liver as described in the text. Each value is the average value±S.D. obtained from three rats.

non-parenchymal cells was seven times greater than that by hepatocytes from liposomes having α-mannoside on the surface whereas the uptake of radioactivity by hepatocytes was three times greater than that by non-parenchymal cells from liposomes having β-galactoside on their surface. Competition experiments using asialofetuin and mannan indicated that the uptake of asialoganglioside liposome entrapped γ-globulin by hepatocytes was inhibited by asialofetuin whereas the uptake of α-mannoside liposome entrapped γ-globulin by non-parenchymal cells was inhibited by mannan (Table I). These results are persuasive enough to demonstrate that asialoganglioside liposomes were specifically taken up by hepatocytes as a direct consequence of the specific interactions of exposed galactosyl residues with the hepatic binding protein described by Ashwell and Morell (1974) and α-man-liposomes were specifically taken up by Kupffer cells as a direct consequence of the interactions of exposed mannosyl residues with the hepatic mannose binding protein (Stahl & Schlesinger, 1980). The present study reveals that liposomal surface β-galactoside and α-mannoside can mediate selective targeting of liposome entrapped biologically active molecules towards different cell types of liver.

Considering the efficacy of glycosylated liposomes in the targeting towards specific liver cell types it was further thought to be of great interest to see whether therapeutic substances enclosed in these types of liposomes can be effective in reversing the diseased conditions of liver. For this purpose galactosamine-induced hepatitis in rats was chosen as the model disease since it is a type of liver injury which closely resembles human viral hepatitis and the severity and duration of this disease can be experimentally controlled by the amount of D-galactosamine (Decker, 1975; and Keppler, 1975). The basic biochemical lesions produced by D-galactosamine in the course of induction of liver cell injury are a deficiency of UTP and an accumulation of UDP-hexosamines which finally leads to a disturbance in RNA and protein synthesis (Decker, 1975; and Keppler, 1975). Due to the high activity of uridine kinase, administration of uridine to D-galactosamine treated rats raises the content of UTP and consequently can reverse the effect of galactosamine-toxicity. Deficiency of UTP also causes a severe depletion of glycogen level and therefore, the course of D-galactosamine induced hepatitis was monitored by the estimation of liver glycogen, which has been shown to be reduced to less than 5 per cent of the controls by a single injection of D-galactosamine (*loc cit.*). Thus D-galactosamine and uridine offer a suitable model system for studying the efficacy of different liposomes in delivering therapeutic substances towards the cells of rat liver. Earlier works from our laboratory have shown that the effective dose of phosphatidic acid liposome-entrapped uridine required for the reversal of this experimental hepatitis was much less than that of free uridine (Mathias *et al.*, 1977). Since there is a selective uptake of glycosylated liposomes by different liver cell types, a comparative study has been undertaken on the protective effect of uridine entrapped in liposomes having different glycosides on their surface and that entrapped in phosphatidic acid containing liposomes.

The rats were first injected intraperitoneally with D-galactosamine (150 mg/kg body wt) and then after 30m free uridine and uridine entrapped in different liposomes were given intravenously. Rats were sacrificed and glycogen level of the liver was estimated after 3h of D-galactosamine administration. Uridine was entrapped in

different glycosylated liposomes and phosphatidic acid liposome which were prepared according to the method described earlier (Ghosh & Bachhawat, 1980 *a, b*). In the competition studies, 10mg of asialofetuin was administered intravenously before injection of liposomes. In this study, the dose of liposome-entrapped uridine was fixed at 80mg/kg body wt. since the maximum possible amount of liposome that can be administered per rat cannot entrap more than the specified dose of uridine. Free uridine given to D-galactosamine treated rats in high dosage (1.28 gm/kg body wt) completely reverse the D-galactosamine toxicity. Table II shows the effect of various types of liposome-entrapped uridine (80 mg/kg body wt) on the liver glycogen level of D-galactosamine treated rats. The liposome-entrapped uridine was found to be more effective in reversing the D-galactosamine toxicity compared to free uridine at the same dosage. It is evident from Table II that asialoganglioside

TABLE II

Effect of various types of liposomes entrapped uridine on the liver glycogen level of D-galactosamine treated rats

Experiments	Liver glycogen level (mg/g liver)
Normal value	45.0±5.0
D-Galactosamine alone	2.2±1.1
Free uridine	5.8±2.1
Dicetylphosphate liposome	4.3±2.1
Phosphatidic acid liposome	12.5±2.5
Asialoganglioside liposome	19.1±3.1
α-man-liposome	7.5±2.2
Asialoganglioside liposome+Asialofetuin	12.2±2.3
Phosphatidic acid liposome+Asialofetuin	11.5±1.2
Asialoganglioside liposome (oral feeding)	4.9±2.0

Female rats, weighing 75–90 gm, in groups of 5 rats were given either free uridine or uridine entrapped in liposomes through the tail vein (80 mg/kg body wt), 30 min after the D-galactosamine challenge (150 mg/kg body wt). The animals were sacrificed at the end of 3h after the D-galactosamine injection and their liver glycogen levels were estimated. The values are expressed as mean±S.D. Each value, when compared with that when D-galactosamine alone was given, is found to be statistically significant ($P<0.01$–0.001).

liposome and phosphatidic acid liposome-entrapped uridine produces more regenerating effect compared to other liposomes studied. At this dose, asialoganglioside and phosphatidic acid liposome-entrapped uridine causes 50 per cent and 30 per cent repletion of liver glycogen level respectively. The regenerating effect of α-mannoside-liposome-entrapped uridine is slightly higher than free uridine at the same dosage. The more protective effect of asialoganglioside liposome-entrapped uridine on the D-galactosamine treated rats compared to α-mannoside liposome indicates that the asialoganglioside-liposomes are taken up more by the parenchymal cells of liver as a direct consequence of the specific interaction of the exposed galactosyl residues with the galactose binding protein present on the parenchymal cells as described by Ashwell and Morell (1974). The inhibition of the protective action of asialoganglio-

side liposome-entrapped uridine by asialofetuin further confirms this observation. The difference in the regenerating effect of phosphatidic acid liposome and asialoganglioside-liposome-entrapped uridine on the D-galactosamine toxicity may be due to the fact that the negatively charged liposomes are taken up by both parenchymal and nonparenchymal cells of liver (Grigoriadis & Ryman, 1972) thus diminishing the effectiveness of uridine whereas asialoganglioside liposomes are predominantly taken up by the liver parenchymal cells which are mainly affected by D-galactosamine (Hofmann *et al.*, 1976). Therefore, it may be suggested that liposomes having β-galactoside on their surface may provide an effective way of delivering therapeutic substances of biological interest to diseased liver as in the cases of viral induced hepatitis, hepatoma, etc.

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Bioorganic Chemistry

A SYNTHETIC APPROACH TO THE PROBLEM OF STRUCTURE-ACTIVITY RELATIONSHIP IN THE FIELD OF GIBBERELLINS

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The paper presents a detailed analysis and discussion of a synthetic approach to the structure-activity relationship in the field of gibberellins, with respect to: the site of primary action of the hormone in a given plant; the fate of a given gibberellin moving from the site of application to the site of action; and how the specific receptors and carriers of gibberellins work.

Keywords: Gibberellins; Structure-Activity Relationship; Hormonal Regulation in Plants

INTRODUCTION

THE knowledge of relationship between the structure and plant growth promoting activity of gibberellins is important for a better understanding of hormonal regulation in plants. In the prospect it may be of great value for agricultural practice. The problem of structure-activity relationship has many aspects and its solution requires a complex approach. To have a full picture of this relationship we ought to know (i) the site of primary action of the hormone in a given plant; (ii) the fate of a given gibberellin as it moves from the site of application to the site of action; (iii) whether there are specific receptors and carriers of gibberellins, and, if so, how they work. Each of these questions is already complex enough so that the full solution of the structure-activity problem can be obtained by way of co-operation between plant physiologists, biochemists and organic chemists.

ANALYSIS AND DISCUSSION

A simplified kinetic scheme of hormonal action (Fig. 1) implies that either at the first or at the second stage, or at both, the binding of gibberellins with specific cellular receptors must take place, the efficiency of this hormone-receptor contact being dependent on the structure of the applied gibberellin. Recently, there appeared reliable data about the specific and reversible binding of gibberellins with cytoplasmic proteins in the responsive tissues of such plants as barley, wheat and pea (Nadeau & Rappaport, 1974; Jelsema *et al.*, 1977; Stoddart *et al.*, 1974; and Konjevic *et al.*, 1976). Moreover, competitive studies show, that the most active gibberellins have greater affinity to these proteins than the weakly active gibberellins. These findings

suggest that we can discuss the structure-activity relationship in terms of molecular interaction. At the same time they stimulate another approach to the structure-activity problem which may provide a basis for the prognostication of growth promoting activity.

This approach consists in the partial synthesis and systematic biological testing of some specially chosen gibberellin analogs derived from the most active natural gibberellins by selective modifications at certain parts of the molecule. Such minor and selective modifications would help estimate the importance of a given part of the molecule for the display of growth promoting activity. The choice of synthetic targets can be made on the assumption that an efficient contact between a gibberellin and a specific receptor is necessary for an efficient growth-triggering reaction to occur.

Now, from the analysis of the literature about the growth promoting activity of gibberellins (Brian, *et al.*, 1967; Agnistikova *et al.*, 1974; Reeve & Crozier, 1975; Hoad *et al.*, 1976; and Sponsel *et al.*, 1977), particularly in three standard bioassays on the seedlings of dwarf pea, cucumber and lettuce one can draw the following conclusions: (*i*) High activity is displayed only by those C_{19}-gibberellins which have a high degree of topological correspondence with the specific receptor; (*ii*) The activity of C_{20}-gibberellins depends on their ability either to metabolize quickly to the active C_{19}-GAs under the conditions of the bioassay or to mimic the structure of these active C_{19}-GAs. For the dwarf pea test and for the cucumber test the structure-activity analysis suggests that the optimal hormone-receptor contact is attained in the structure of GA_3 or in the structure of GA_7, respectively (Fig. 1).

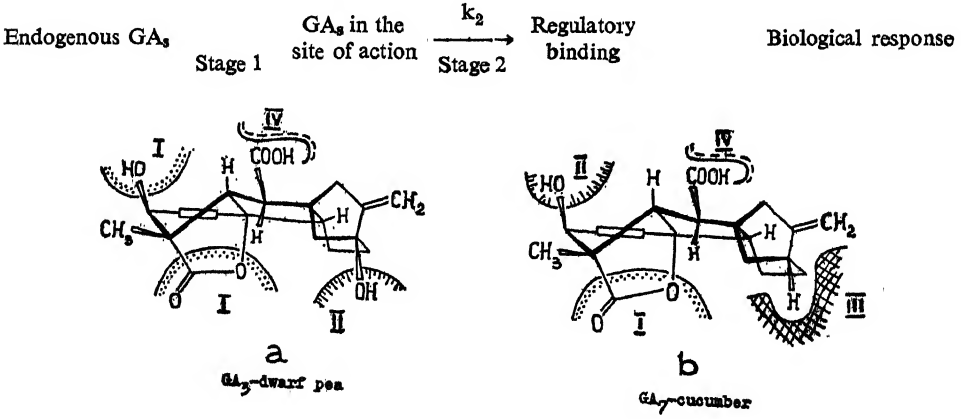


FIG. 1. Schematic representation of the hypothetic optimal contact between a gibberellin and a GA-specific receptor. I. Obligatory binding. II. Ancillary binding. III. Hydrophobic locus. IV. Ionic binding(?).

With this picture in mind we started the synthesis of close analogs of gibberellins A_3 and A_7 in order to check whether the results of bioassays will be compatible with the forecasts made on the basis of the hypothesis about the importance of the hormone-receptor contact for the display of activity. For the synthesis of analogs with

sufficient potency it was necessary to use such designs which would leave the C-7 carboxyl group untouched or allow its regeneration under possibly mild conditions.

In order to appreciate the role of the free carboxyl group, the synthesis of 7-homo-gibberellin A_3 from GA_3 was carried out as outlined in Fig. 2, the key step being the photo-induced Wolff rearrangement (Serebryakov *et al.*, 1978 *a*). 7-Homo-GA_3 (**4**) can also be obtained by a multi-step synthesis involving the iodide prepared earlier by Lischewsky and Adam, but the yield is very poor.

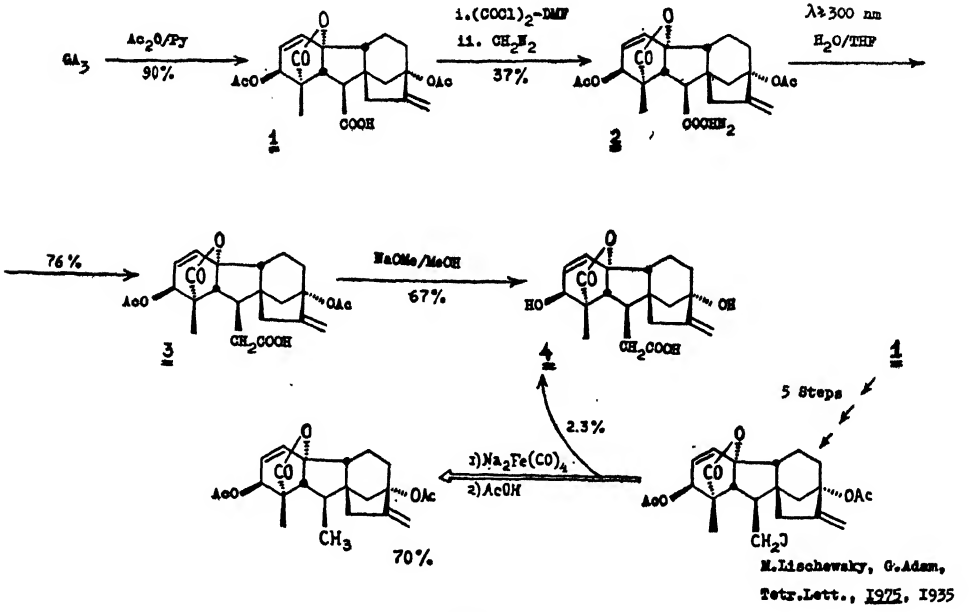
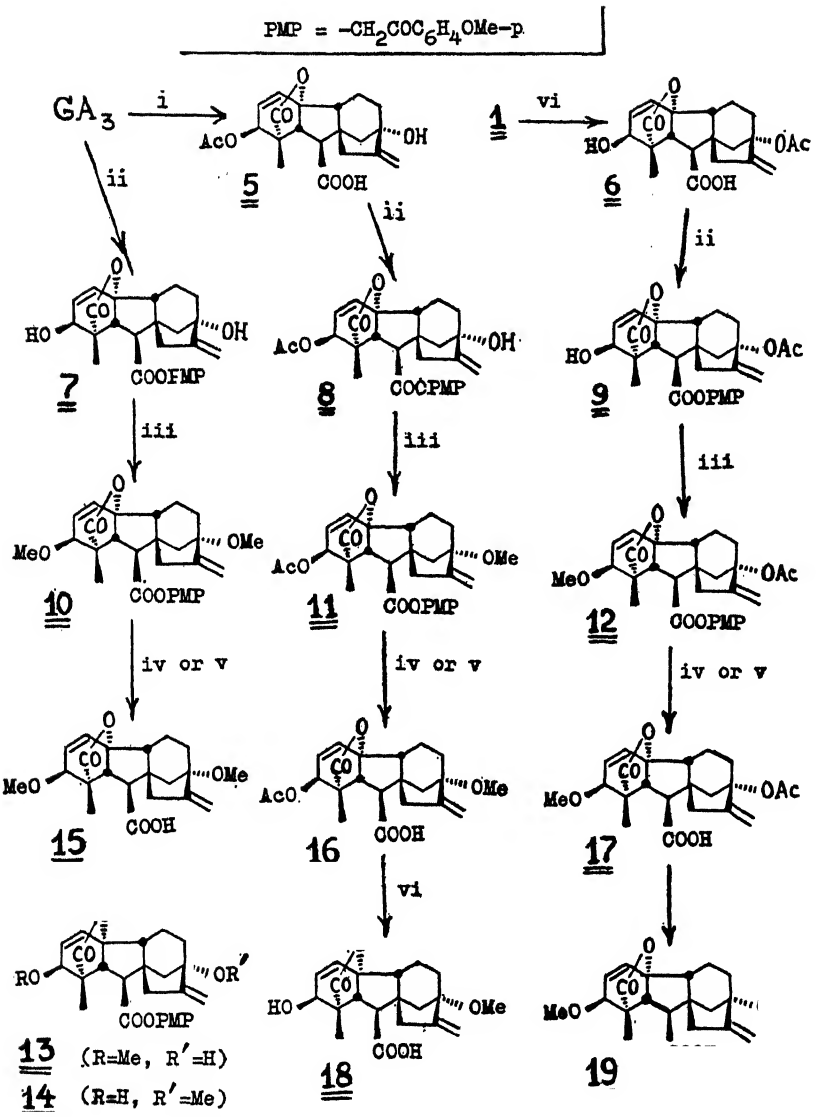


FIG. 2

In order to appreciate the relative importance of the alcoholic hydroxy groups in GA_3, they were transformed in the corresponding methyl ethers (Fig. 3). Since the enzymes cleaving the ether linkage are not likely to operate in plants, this modification seems to exclude the possibility of the hydrogen bond formation between the hormone and the receptor. For the synthesis of all three O-methyl analogs of GA_3, we made use of the light-sensitive p-methoxyphenacyl group as a protection of the carboxyl function (Serebryakov *et al.*, 1978 *b*); this protection can also be removed by a conventional reductive way. Interestingly, the methylation of the tertiary OH group in (**8**) proceeds better than that of the secondary OH group in (**9**); moreover, the methylation of the dihydroxy ester (**7**) affords a mixture of side products (**13**) and (**14**) in which the latter predominates.

This preference for the alkylation of the tertiary OH group may be explained by the relative ease with which the Ag^+ ion is co-ordinated to the exocyclic double bond of the ring D in comparison with the endocyclic double bond in the ring A (Fig. 4).

In a similar way we obtained 3-0-methyl-GA_7 (**22**) (Serebryakov *et al.*, 1981). Starting from the ester (**9**) we obtained also the 3-0-glucoside of GA_3 (**27**); in this



Reagents: *i.* Ac_2O/Py; *ii.* PMP—Br/NEt_3/DMFA(46–83%)
iii. CH_3J/Ag_2O/THF(48–79%); *iv.* hν/abs.EtOH(36–62%);
v. Zn/AcOH(50–70%; *vi.* O.02N NaOMe/MeOH(54–57%).

FIG. 3

sequence (Fig. 4) the photolytic cleavage gives the desired intermediate **(26)** in 42 per cent yield while deprotection with Zn/AcOH gives mainly 13-0-acetyl-GA_3 **(6)**. This example shows the advantage of the photolytic deprotection when a molecule contains acid-sensitive groups. Again, the glucosylation of the dihydroxy ester **(7)** gives a mixture of monoglucosylated products with predominance of the tertiary tetra-acetyl glucoside **(24)**.

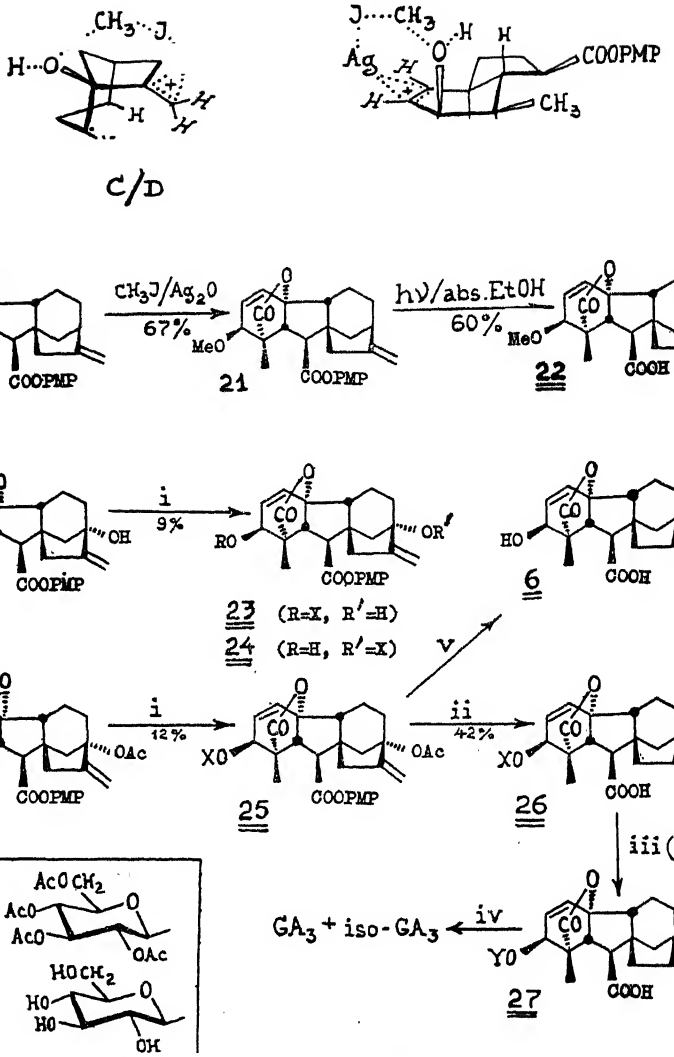
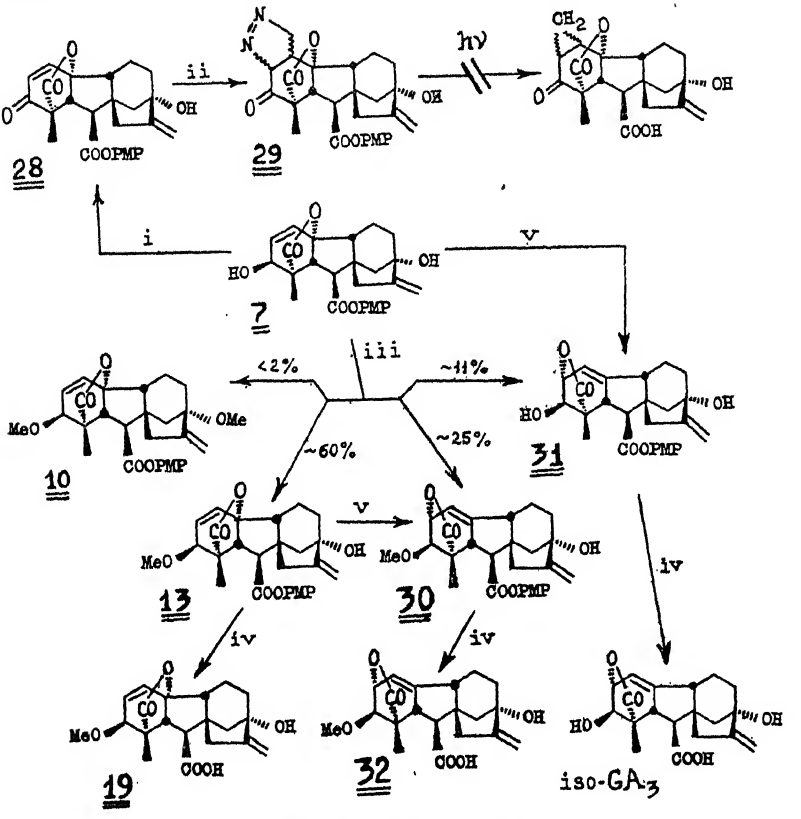


FIG. 4

Although the PMP protection easily survives the conditions of the manganese oxidation — and this opens the way to the pyrazoline **(29)** — the latter could not be converted to the desired cyclopropane containing acid neither photolytically nor otherwise (Fig. 5). This failure prompted us to try the direct cyclopropanation of the allylic double bond upon reaction with CH_2N_2 in the presence of $Pd(OAc)_2$. No cyclopropanation products were obtained, the reaction product being a mixture of four compounds. The main component of it was identified as the ester **(13)** whose deprotection afforded 3-0-methyl-GA_3 **(19)**. Thus, **(19)** could be obtained from

GA_3 in three steps only instead of the six-steps sequence outlined in Fig. 3. Two other products, **(30)** and **(31)**, result from the double bond migration (Serebryakov,



Reagents: i. MnO_2/acetone; ii. CH_2N_2;
iii. CH_2N_2/$Pd(OAc)_2$; iv. Zn/AcOH ; v. $Pd(PPh_3)_4$

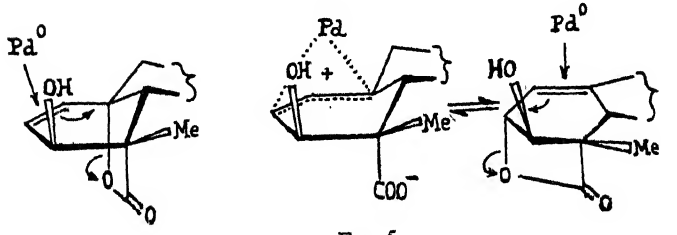


FIG. 5

1980). In spite of the great excess of CH_2N_2, only traces of the dimethylated ester **(10)** were obtained, a hint that palladium coordinates predominantly with the endocyclic rather than exocyclic double bond. In the abscence of CH_2N_2 the migration of the Δ^1-double bond cannot be induced by Pd (OAc) alone. Probably, this isomerization is due to the traces of zero-valent palladium formed in the reduction of Pd^{2+} by CH_2N_2. Indeed, when the PMP esters **(7)** or **(13)** were treated with the

soluble Pd $(PPh_3)_4$ complex they afforded the corresponding iso-compounds, **(31)** or **(30)**, in high yield. This isomerization can be explained by a two-step mechanism where the lactone bridge plays in turn the role of a leaving group and of a nucleophile and where the equilibrium between two isomers is attained through a π-complex (cf. Trost, 1977).

When we tried to make use of the palladium-catalyzed decarboxylation of allylic formates (Hey & Arpe, 1973) for the preparation of 3-desoxy-GA_3 (Fig. 6), the decarboxylation of 3-0-formyl-GA_3 **(33)** gave not the desired product but a

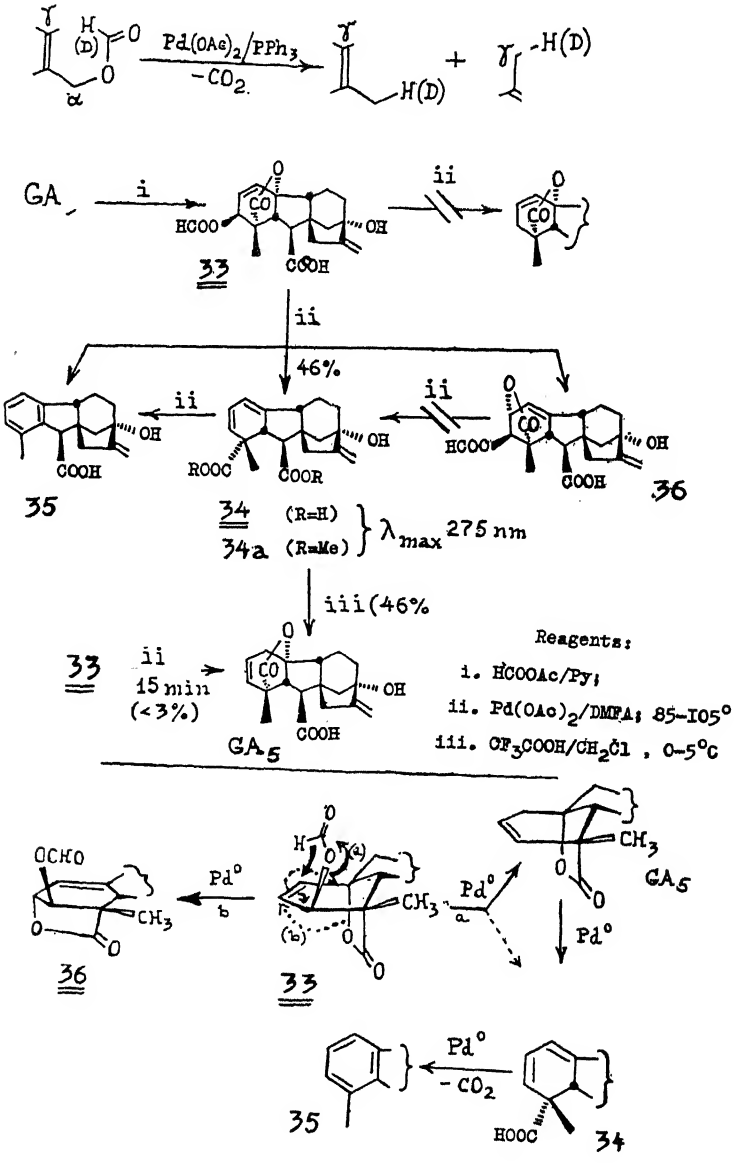
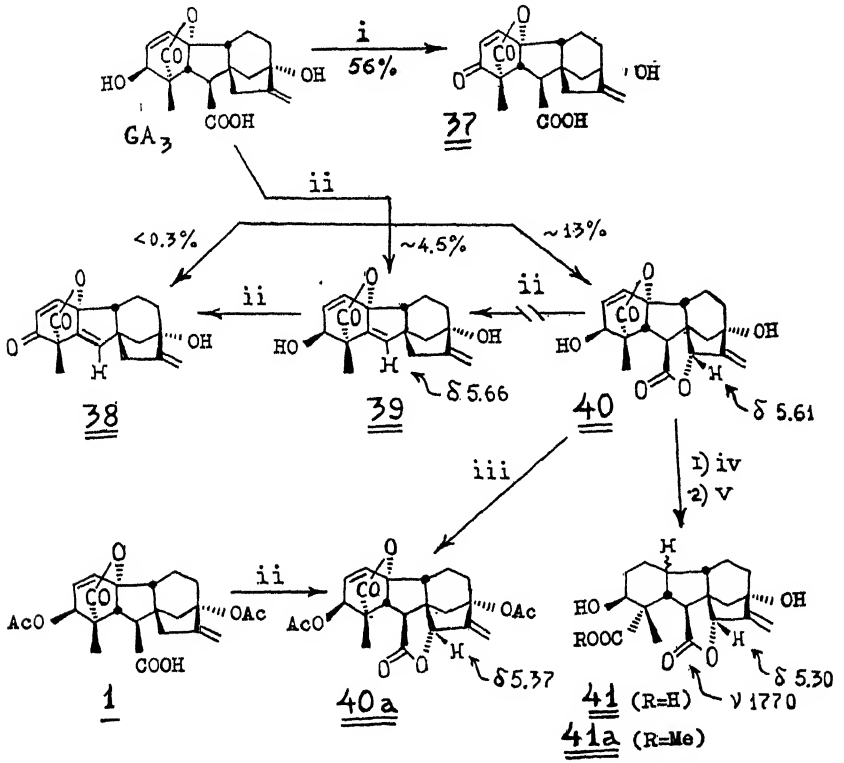


FIG. 6

dicarboxylic acid (**34**) with a homo-annular array of conjugated double bonds. Epiallogibberic acid (**35**) and iso-GA_3 monoformate (**36**) were obtained as side products. The structure of (**34**) was confirmed by its easy transformation into gibbrellin A_5 under non-aqueous mildly acidic conditions. If the reaction of (**33**) with Pd $(OAc)_2$ + PPh_3 is carried out for a short time then GA_5 can be obtained as the primary product albeit in very low yield. The tentative mechanism of formation of all products in this reaction is shown in Fig. 6.

3-Dehydro-GA_3 (**37**) appears to be a useful intermediate for the synthesis of modified GA_s. The best yields of (**37**) were obtained upon oxidation of GA_3 with



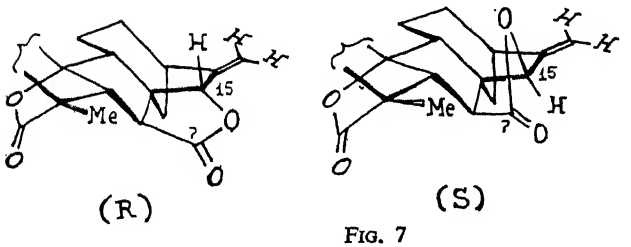


FIG. 7

the "basic" MnO_2 prepared according to Attenborough *et al.* On the other hand, if the oxidation of GA_3 is carried out with the "neutral" MnO_2 prepared according to Mancera *et al.*, the reaction gives only 5–6 per cent yield of the enone acid and the recovery of GA_3 exceeds 50 per cent even after 8 days of oxidation at room temperature. At the same time three neutral products are obtained (Fig. 7): a nor-enone **(38)**, a noralcohol **(39)** and a dilactone **(40)**, the latter being the main component of the mixture (Kobrina *et al.*, 1973). The position of the newly formed lactonic bridge follows from the spectral evidence and from the selective hydrogenolysis of **(40)** under the conditions preventing the attack on the Δ^{16}-double bond. Since the

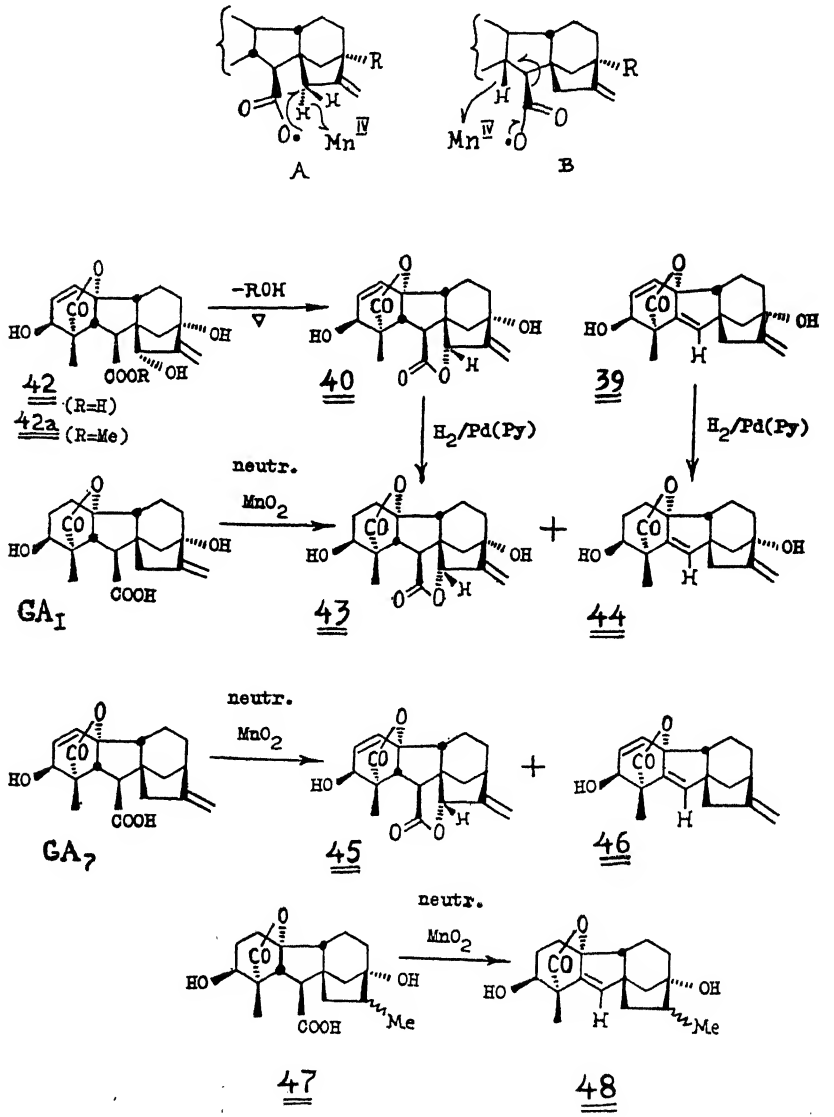


FIG. 8

manganese oxidation of GA_3-diacetate **(1)** affords the dilactone diacetate **(40a)** it is clear that the oxidation with "neutral" MnO_2 involves only the carboxyl group. From the consideration of molecular models (Fig. 7) it was concluded that the stereochemistry at C-15 in the dilactone **(40)** must be (R) and not (S), a conclusion that was later confirmed by the X-ray studies (Kutschabsky *et al.* — *In Press*).

Since the nor-olefin **(39)** cannot be obtained from **(40)** by oxidation with "neutral" MnO_2, the formation of **(39)** and **(40)** must result from two parallel reactions.

It is tempting to think that **(40)** and **(39)** represent two ways of stabilization, A or B, of the same carboxy radical formed on the surface of MnO_2 (Fig. 8). However, if the oxidation of GA_3 is carried out with under-dried "neutral" MnO_2, then a trihydroxy acid **(42)** can be occasionally obtained in low yield. Both **(42)** and its methyl ester **(42a)** are very unstable and easily produce **(40)** even on storage.

The general character of the anomalous oxidation of free GA_s with "neutral" MnO_2 was demonstrated (Serebryakov & Kucherov, 1976) in reactions with GA_1 and GA_7 (Fig. 8). The oxidation products obtained from GA_1 were correlated with those obtained from GA_3 by selective hydrogenation on the poisoned Pd catalyst. Interestingly, the oxidation of GA_3, GA_1 and GA_7 gives nearly the same ratio of dilactone to nor-olefin (ca. 3:1). The presence of the Δ^{16}-double bond is necessary for the oxidative lactonization to occur, the only product from the oxidation of tetrahydrogibberellin A_3 being the nor-olefin **(48)**.

Dilactones **(40)** and **(43)** are less sensitive to the acidic hydrolysis than their respective precursors, GA_3 and GA_1 (Fig. 9). The hydrolysis of **(40)** affords a hetero-

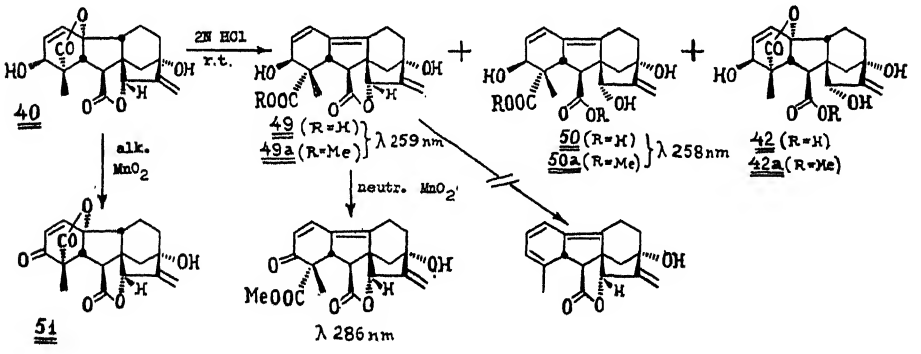
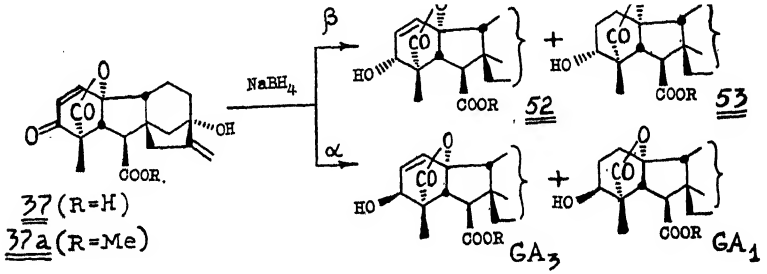


FIG. 9

annular diene lactonic acid **(49)** as the main product accompanied by acids **(50)** and **(42)**, but, unlike the hydrolysis of GA_3, no detectable amount of the ring A aromatization products (Voigt *et al.*, 1977 *a*). It is interesting to compare the hydrolysis of **(40)** with the hydrolytic aromatization of GA_3 for which the formation of a conjugated triene system as the necessary intermediate is postulated. Apparently, in the case of **(40)** the analogous intermediate cannot be formed because of the additional strain imposed by the 7 → 15 lactone bridge. Similarly, on heating with diluted HCl dilactone **(43)** remains unaffected while GA_1 undergoes Wagner-Meerwein rearrangement to give gibberellin C. The conformational influence of the dilactone system can be seen also in the fact that the ester **(49a)** is oxidized by the neutral MnO_2 while the dilactone **(40)** affords the corresponding enone **(51)** only with the more active basis, MnO_2.

The preparation of 3-epi-GA_3 **(52)** by the borohydride reduction of the enone acid **(37)** is necessary for the estimation of the influence exerted by the configuration of the 3-OH group on biological activity (Fig. 10). The best yield of **(52)** — up to



Stereochemistry of the hydride reduction

Reagent	Molar ratio enone : hydride (37a)	Solvent	Main producets (yield in %%) 52a	53a
$NaBH_4$	1 : 1.08	Dioxan — H_2O	17	12
$NaBH_4$	1 : 1.05	MeOH	10	20
$LiBH_4$	1 : 1.25	abs.THF	—	50
$Zn(BH_4)_2$	1 : 1.50	abs.THF—ether	24	25

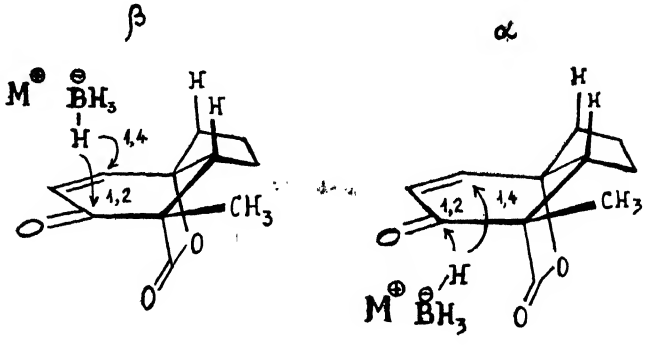


FIG. 10

35 per cent was obtained with large excess of $NaBH_4$ in MeOH, the side products being 3-epi-GA_1 (53), GA_3 and GA_1 (Voigt *et al.*, 1977 *b*; Gurvich *et al.*, 1969; and Beale & Macmillan, 1980). The reduction of the ester (37a) with complex borohydrides strongly depends on the conditions but in all cases the main products, isolated upon chromatography of the four-component mixture, corresponded to the addition of the hydride anion from the β-face of the enone system. The α-face of the enone seems to be somewhat more hindered (Fig. 10).

The same stereoselectivity was observed in additions to the enone double bond. The reactions shown on Fig. 11 may be relevant to a plausible mechanism of

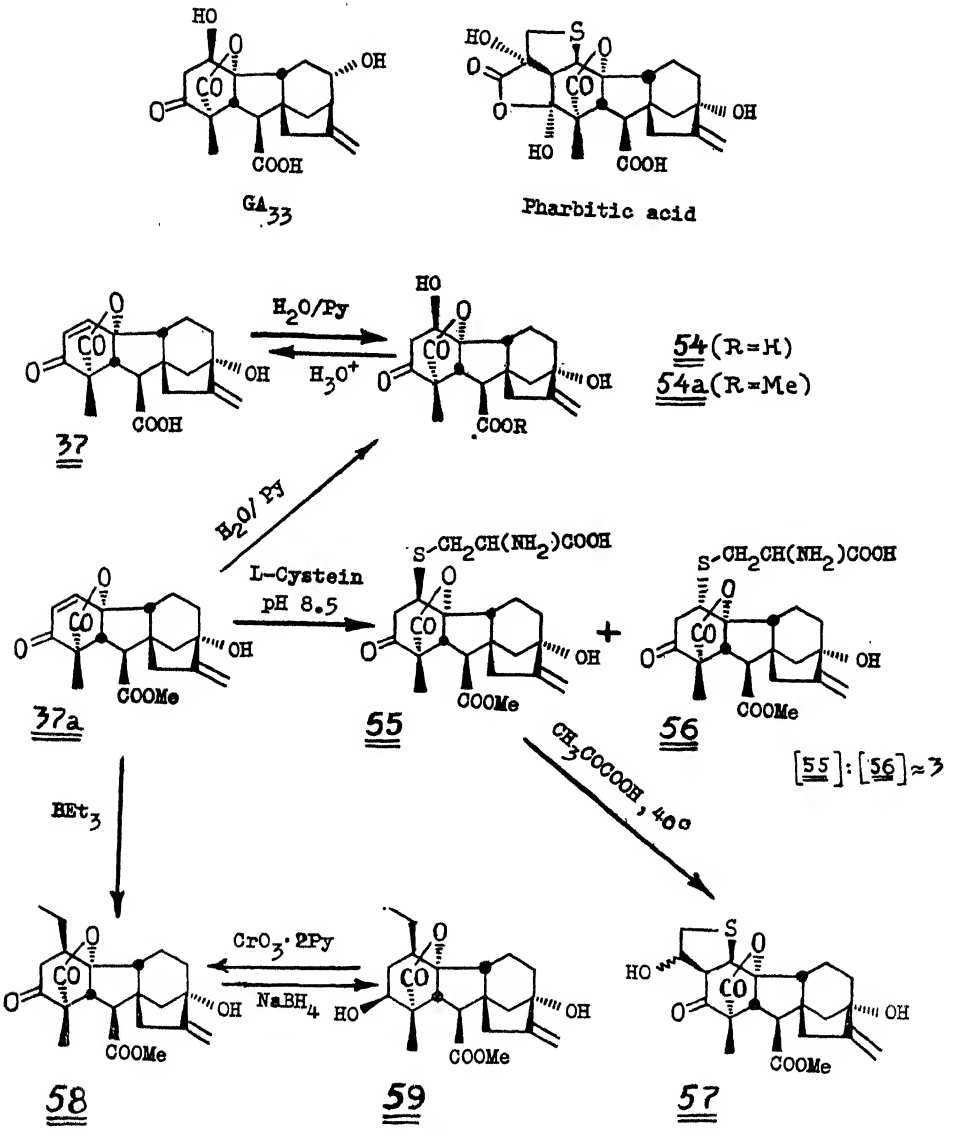
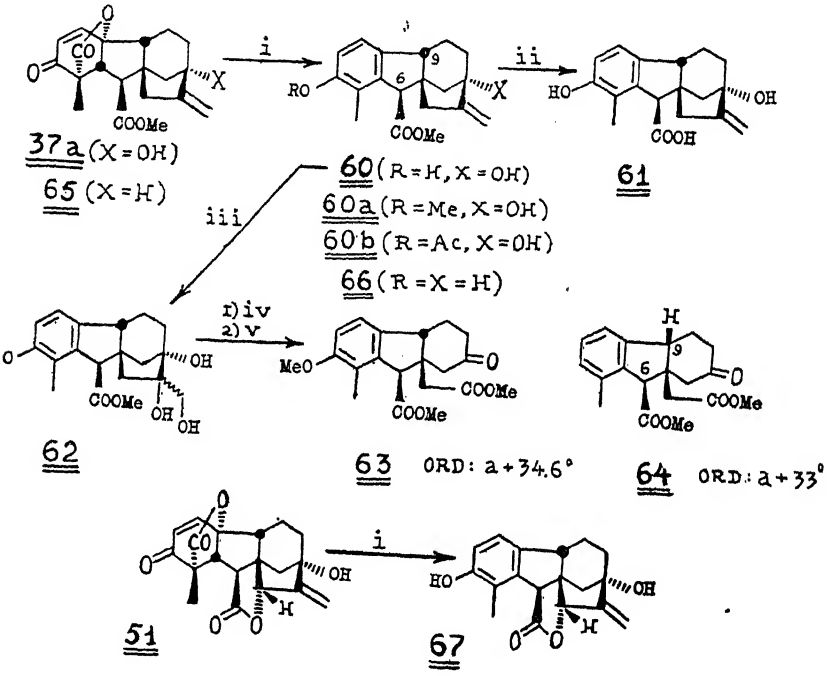


FIG. 11

inactivation of GA_3 or GA_{30} in Convolvulaceae, where the formation of the nearly inactive GA_{33} or pharbitic acid seems to be due to some Michael-type addition of water or cystein to the corresponding enones. The hydration of **(37)** or **(37a)** in pyridine at room temperature proceeds stereoselectively (Serebryakov *et al.*, 1974) to give 1 β-hydroxy-3-dehydro-GA_1 **(54)** or its ester **(54a)** which are close analogs of GA_{33}. The reaction of **(37a)** with cystein also proceeds with predominant formation of the (1R) — isomer **(55)**. Upon the decarboxylative trans-amination **(55)** affords a ketol closely related to pharbitic acid. Such free-radical agent as triethylborane



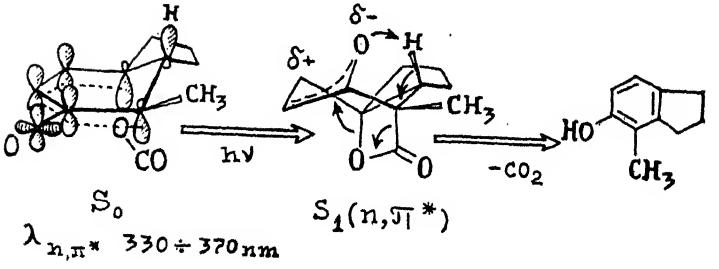


FIG. 12

also reacts with **(37a)** to give mainly 1 β- ethyl derivative **(58)**. The stereochemistry of these 1-substituted 3-oxogibberellins was determined by combination of NMR and DC methods (*vide* infra).

The photolysis of **(37a)** in tert-BuOH (or in benzene) gives a tetracyclic phenol **(60)**, from which the corresponding acid **(61)** and derivatives **(60a, b)** were obtained (Fig. 12). The stereochemistry of **(60)** at C-6 and C-9 was proved by its conversion to the tricyclic keto diester **(63)** with nearly the same ORD curve as that of the keto diester **(64)** derived from epiallogibberic acid (Gurvich *et al.*, 1971). The same photo-induced decarboxylation-aromatization was observed upon the photolysis of enones **(51)** (Voigt *et al.*, 1975) and **(65)**, the yields of all three phenols being within 30–42 per cent. Since the stereochemistry at C-6 and C-9 is not altered by the photo-aromatization the latter is likely to proceed as a concerted elimination of the lactone bridge. The formation of phenols is not quenched by oxygen or conjugated dienes and hence proceeds *via* a singlet state. From the UV spectrum of **(37a)** it follows that there must be a strong overlap between the σ-orbitals of the lactone bridge and the enone π-system. The stereochemistry of the enone also favours the concerted decarboxylation-lactonization (Fig. 12).

The photolysis in hydrogen-donating solvents (Fig. 13) proceeds differently (Serebryakov *et al.*, 1972*a, b*). When photolyzed in benzyl alcohol, which is a good hydrogen donor, **(37a)** gives a tricyclic phenol **(68)** whose structure was deduced from the spectra of its derivatives. In the NMR spectra of **(68)** and **(68b)** the signal of the benzyl proton (formerly at C-9) appears as a distinct C-part of an ABC-system, the breadth of which corresponds to the retention of configuration at this centre (assuming that the NMR spectra are due to the more stable conformer in the boat-chair equilibrium). Phenol **(68)**, obtained in 40 per cent yield, is accompanied by the ring A-saturated ketone **(69)** and by traces of the tetracyclic phenol **(60)**. Since the latter does not produce **(68)** upon photolysis in benzyl alcohol, this tricyclic phenol must be formed by an independent mechanism.

The photolysis in aliphatic alcohols is more complex (Fig. 13). In ethanol both O-addition and C-addition of the solvent takes place, the stereochemistry of the isolated products, **(70)** and **(71)**, being as shown. Moreover, the same products as in the case of photolysis in benzyl alcohol were obtained. The photolysis in isopropanol gives qualitatively the same results. The stereochemistry of the side chain in the C-adduct **(71)** was established by the sequence **(71)** → **(74)** → **(75)** → **(76)**. Compound **(76)** thus obtained had the configuration at C-1 opposite to that in the enone-triethylborane adduct **(58)**.

The photolysis of the epoxy derivative of the enone **(37a)** in dioxane gives a tetracyclic resorcinol **(77)**, characterized by its dimethyl ether **(77a)**. At elevated temperature it gives in addition the known hydroxy ketone **(54a)** while the yield of **(77)** remains unaffected. The formation of **(77)** is believed to arise from the well-known epoxy ketone-β-diketone rearrangement with subsequent decarboxylative aromatization of the enol tautomeric form.

Until now, the photo-induced aromatization of ring A represents the only successful way to the biologically interesting phenolic derivatives of gibberellins. Attempts to introduce phenolic function by conventional means (Mori *et al.*, 1969; and Adam & Hung, 1974) gave the phenolic products with rearranged rings C and D.

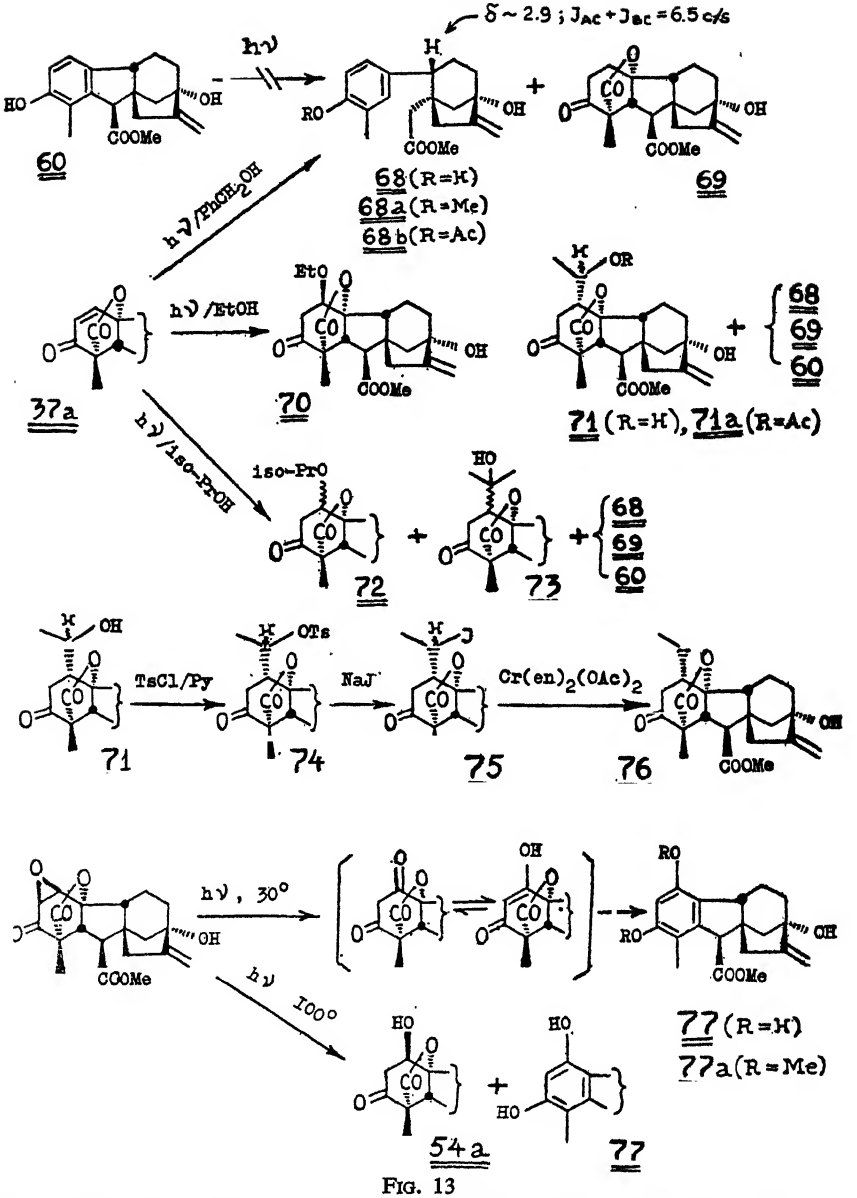


FIG. 13

The mechanisms of all photochemical transformations outlined in Figs. 12 and 13 were elucidated by quenching experiments. All types of aromatization and the formation of O-adducts proceed through a singlet state, while the reduction of the enone double bond or epoxide and the formation of C-adducts (as well as the ubiquitous and extensive cyclodimerization of the enone system) take place from a triplet state.

The photocycloaddition of enones (37) and (37a) to HC ≡ CH gives the cycloadducts (78) or (78a) in modest yields (Serebryakov *et al.*, 1977*a*, *b*). As by-products

of this sluggish reaction phenols (61) or (60) and traces of a 1:2 adduct were obtained (Fig. 14). The stereochemistry of (78) and (78a) follows from the negative Cotton

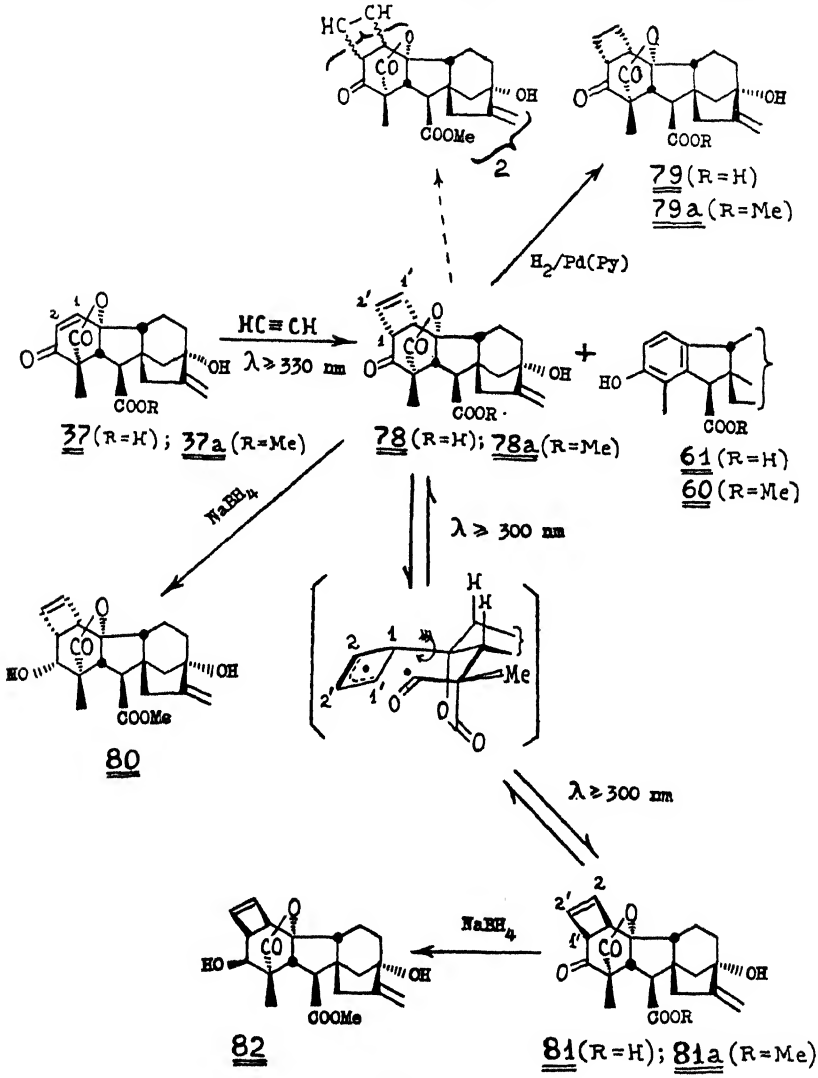


FIG. 14

effect in their CD-spectra and from their selective hydrogenation to the previously described (Voigt & Adam, 1976) cyclobutane (79) with known stereochemistry. Compounds (78) and (78a) are the only 1:1 adducts when addition is induced by the light with $\lambda > 330$ nm, that is, by the light absorbed by the starting α, β-enone. When reaction is carried out simply in Pyrex ($\lambda > 300$nm) which does not prevent the excitation of the β, γ -enone system, then mixtures of 1α, 2α -cycloadducts with their 1β, 2β -isomers, (81) or (81a), are obtained in ca. 3:2 ratio. The borohydride reduction of (78a) and (81a) gives mainly the alcohols, corresponding to the attack from the less hindered side.

From the consideration of molecular models of 3-dehydro-GA_1 methyl ester **(69)** it follows that the chair conformation of ring A must be more favourable than the boat conformation at least by 0.9 kcal/mole. All substituents in the 1β -position tend to decrease the stability of the chair conformation. Although the chair form still prevails when the substituent is heteroatomic and small (OH, OMe or SMe, all having $-\Delta G°$ below 0.70 kcal/mole), its contribution should be considerably reduced in the case of bulkier alkyl substituents (for Et, $-\Delta G° = 1.75$ kcal/mole). In the case of 1α -substituted derivatives the chair form is always more favoured. From these considerations it follows that the configuration at C-1 may be safely deduced from the breadth of the H_x signal splitting in compounds with heteroatomic substituents. But when the substituent at C-1 is carbon, its orientation cannot be ascertained so simply. However, in such cases a correlation between the NMR and CD data of 1-substituted 3-oxogibberellins may be helpful (Fig. 15).

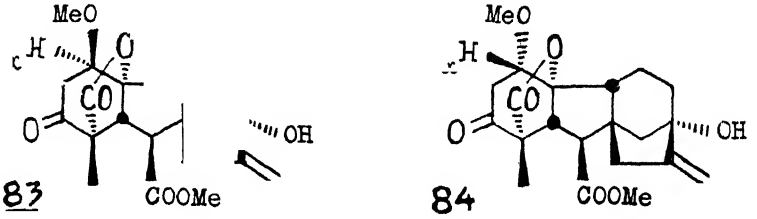
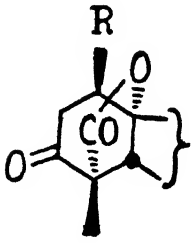
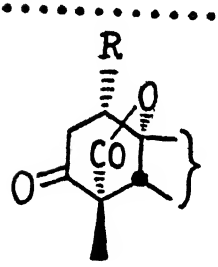


FIG. 15

Correlation between the data of ^{1}H-NMR and CD spectra of 1-substituted 3-dehydro-GA_1 methyl esters

Ketone type	Compounds (*R*)	$J_{AX} + J_{BX}$ (*c/s*)	CD (n,π^*), degr (Θ)	a	Orientation of the substituent at C-1 NMR	CD
	**69** (R=H)	—	+11800	+144	—	—
	83 (R=OMe)	7	+12000	+146.8	*a*(β)	*a*(β)
	54 (R=OH)	7	+10800	+132	*a*(β)	*a*(β)
	55 (R=·S-Cysteyl)	7	+11400	+138	*a*(β)	*a*(β)
	58 ($R=\cdot C_2H_5$)	—	+7500	+91.5	—	*a*(β)
	70 (R=·OEt)	7	+13150	+148	*a*(β)	*a*(β)
	**84** (R=OMe)	12	+14800	+180	*e*(α)	*e*(α)
	56 (R=·S-Cysteyl)	14	+13700	+167	*e*(α)	*e*(α)
	71 ($R=\cdot CHCH_3$, OH)	—	+14400	+175.8)	—	*e*(α)
	76 ($R=\cdot C_2H_5$)	—	+14600	+178	—	*e*(α)

The octant rule predicts for the chair conformation of 3-oxo gibberellins a big positive Cotton effect while for the boat conformation it can be only slightly positive. If so, the greater is the part of the chair form in the conformational equilibrium, the greater must be $[\theta]_M$. To check this we compared the NMR and CD data of two model compounds, **(83)** and **(84)**, obtained in our laboratory (Serebryakov *et al.*, 1972 *b*). In accordance with the above forcast the isomer with small $/ J_{AX} + J_{BX} /$ value, that is, 1 β-substituted ketone, showed smaller $[\theta]_M$ value than its counterpart with greater $/ J_{AX} + J_{BX} /$, where the chair form is sure to prevail (Fig. 15). As can be seen from the table in Fig. 15, there are two groups of 1-substituted 3-oxo gibberellins: those with small NMR splitting and relatively small $(\theta)_M$ values (1β-substitution) and those with larger NMR splitting and larger $[\theta]_M$ values (1α-substitution).

The results of standard bioassays on seedlings of dwarf pea, cucumber and lettuce were expressed in a quasi-logarithmic scale (Serebryakov *et al.*, 1981). Compounds displaying the highest potency in a given bioassay are marked by 4, compounds, whose potency ranges from 10 to about 99 per cent are marked by 3, those, whose activity lies between 1 and about 9 per cent of the highest are marked by 2, weakly active substances are marked by 1, and those devoid of activity are marked by 0. The general trend is that the more the structure of a derivative deviates from that of the most active natural gibberellins, GA_3 and GA_7, the steeper is the fall of activity. It is interesting to compare the potency of acetates **(1)**, **(5)** and **(6)** derived from GA_3, with the potency of the corresponding methyl ethers **(15)**, **(18)** and **(19)**. For the former, the enzymic hydrolysis in plant tissues is an acceptable possibility, and their potency is close to that of GA_3 itself. Among the latter, high potency is displayed only by compound **(18)** with free 3β-OH group. Epimerization at C-3 decreases the potency by about two orders of magnitude in respect of GA_3. Particularly interesting is a sharp fall of activity on passage from GA_3 to 7-homo-GA_3 **(4)**.

One might argue that the decrease of activity is due to different ability of natural and semi-synthetic gibberellins to penetrate to the site of action. In order to model the passive transport of GA_3 in plants, we determined their partition coefficients at two *p*H values between aqueous buffer solutions and ethyl acetate as lipophile medium. No obvious correlation between lipophility and growth promoting potency could be found; compounds with nearly the same *P* values may differ very sharply by their potencies (e.g. GA_3 and 7-homo-GA_3). Therefore, the passive permeability seems not to be very important for the display of growth promoting activity. Topological correspondence between the hormone and the recognizing part of a specific receptor may be of greater significance. Thus, in the case of 7-homo-GA_3 **(4)** the binding of the carboxyl to the complementary part of the receptor may require some rotation around the C-6/C-7 bond (Fig. 16) which would worsen the contacts with other parts of the receptor and hence reduce the activity.

Now, if we adopt an approach developed by Free and Wilson, that is, the principle of additivity of contributions made by substituents to the physiological activity of a compound (Craig, 1972), will we obtain a statistically significant correlation between the structure and growth promoting activity of gibberellins?

Both natural and semi-synthetic gibberellins may be presented as a combination of a common framework with different substituents attached to it at indicated posi-

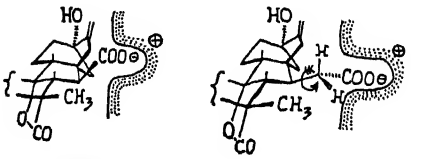
*Relative growth-stimulating potencies of acidic analogs of GA_3 in three standard bioassays**

Compound	Bioassay: Dwarf pea	Cucumber	Lettuce
GA_3	4	2	4
iso-GA_3	2	2	2
GA_7	3	4	4
3-Dehydro-GA_3 (37)	2	0	2
3-epi-GA_3 (52)	2	1	2
1β-OH-3-dehydro-GA_1 (54)	1	0	1
Phenolic acid (61)	0	0	0
1α, 2α-Cycloadduct (78)	0	–	0
7-Homo-GA_3 (4)	1	0	2
7-Homo-GA_3 Diacetate (3)	0	0	1
GA_3 Diacetate (1)	3	2	4
3-0-Acetyl-GA_3 (5)	3	2	4
13-0-Acetyl-GA_3 (6)	4	2	4
3,13-0,0-Dimethyl-GA_3 (15)	1	2	2
13-0-Methyl-GA_3 (18)	3	3	3
3-0-Methyl-GA_3 (19)	2	1	1
7,15-Lacton-19-acid (49)	2	0	1
3-0-Methyl-iso-GA_3 (32)	1	0	1
1(10),2-Diene-7,19-dioic acid (34)	1	0	1
3-O-Methyl-GA_7 (22)	2	3	2
GA_{13}	0	0	1

*From dose-response curves ($10^{-4} \div 10^{0}$ mcg/seedling for dwarf pea and lettuce, $10^{-3} \div 10^{0}$ mcg/seedling for cucumber)

Partition coefficients (P) of GA_3 and its acidic analogs

Compound	P—$[GA_{H_2O}] / [GA_{ACOET}]$	
	*p*H 6.4	*p*H 3.5
GA_3	9.64	0.18
GA_7	0.52	0.02
iso-GA_3	9.85	0.20
3-epi-GA_3 (52)	9.20	0.30
3,13-0, 0-Dimethyl-GA_3 (15)	0.19	0.00
13-0-Methyl-GA_3 (18)	0.95	0.10
3-0-Methyl-GA_3 (19)	0.76	0.09
1β-OH-3-dehydro-GA_1 (54)	9.90	0.22
Phenolic acid (61)	∞	0.16
7-Homo-GA_3 (4)	9.90	0.25



GA_3 4

FIG. 16. Conformational differences between GA_3 and 7-homo-GA_3 in the binding to the GA-specific receptor.

tions A, B, C etc. (Fig. 17). If the activity contribution of hydrogen is assumed to be zero at any position, then for a given GA_s one can write an equation of Free-Wilson type, shown below (Fig 17).

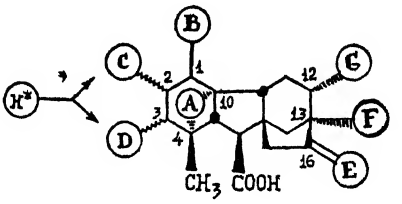


FIG. 17. The Gibberellin framework and the groups of substituents.

Group A: K_1 (-COO-, γ-lactone); K_2 (-$COCH_2$-, δ-lactone); K_3 (-COOCHOH-, δ-lactol).
Group B: K_4 (△α-double bond); K_5 (1β-OH).
Group C: K_6 (2α-OH); K_7 (2β-OH); K_8 ($\triangle^2$ -double bond).
Group D: K_9 (3α-OH); K_{10}(3β-OH); K_{11}(3β-OMe); K_{12} (3 -oxo).
Group E: K_{13} (=CH_2, $\triangle^{16}$-double bond); K_{14} (17-oxo, 17-nor); K_{15} (16β-CH_3); K_{16} (16α-OH); K_{17} (16α-CH_3).
Group F: K_{18} (13α-OH); K_{19} (13α-OMe)
Group G: K_{20} (12α-OH).
Group A: K_{21} (10α-CH_3, 4α-COOH); K_{22} (10α-COOH, 4α-COOH).
Correction factor H^*: K_{23}* (2β-OH: 3β-OH interaction).

Free-Wilson Approach:

$A_j = M + a_1x_1 + a_2x_2 + \ldots\ldots + a_kx_k$

where A_j — relative potency of the j-th compound;
a_k — activity contribution of the k-th substituent;
x_k — fictitious variable (1 or 0);
x — fictituous variable (1 or 0); and
$j = 1,2,3\ldots N$—the oridinal of a given compound (and the number of the corresponding equation).

In this way each compound is described by an equation. Solving the system of such equations by means of the least square method one finds the activity contributions from different substituents as coefficients in the equation. A set including 67 equations (that is, 67 compounds for which the relative potency indices could be presented uniformly on the basis of experimental dose-response curves) and 23 variables (that is, 23 substituents) was solved by the least-square method to give the mathematical activity contributions shown in the Table on p. 40 (Serebryakov *et al.*, 1981). The experimental potency indices give only the order of magnitude but show no difference between compounds belonging to the same group of activity. The indices calculated by summation of framework and substituent contributions in most cases express the activity more accurately. For instance, if we compare the calculated potency indices for the eight best studied gibberellins with their experimentally found activity in the dwarf pea test, the coincidence will be very good ($A_3 > A_7 > A_1 > A_4 \gg A_5 \gg A_{20} \lesssim A_9 >> A_8$).

The statistical analysis shows that the coincidence between calculated and experimentally found values is not accidental but has a regular character. The multiple

correlation coefficients are good for the dwarf pea and cucumber tests ($/R/ \geq 0.90$) and satisfactory for the lettuce test ($/R/ \geq 0.85$). Also, the Fisher's criterion is by far larger than is necessary for a set of 67 equations with 23 variables to prove that the correlation is regular. The values of the explained variance (*EV*) show the mathematical expectation of an accurate prognosis in each bioassay.

Particularly interesting is the hierarchy of activity contributions from the substituents at C-10 taking part in the formation of lactonic bridge: γ -Lactone > δ-Lactone ≥ δ-Lactol ≶ 19,20-di-COOH > 19-COOH, 20-Me, which is compatible with the general trend of gibberellin biosynthesis (Graebe & Ropers, 1978). The lower proportion of successfully predicted potency indices for C_{20} -gibberellins in comparison with C_{19} -gibberellins is apparently due to the interference of metabolic factors.

Mathematical activity contributions from the molecular framework (M) and the substituent (a_k) in the growth-stimulating potency of gibberellins and their analogs

Contributing groups	Bioassay		
	Dwarf pea	Cucumber	Lettuce
Molecular framework (*M*)	—0.18	1.96	—0.48
Ring A, γ-lactone	2.18	1.04	1.48
Ring A, δ-lactone	1.61	0.32	0.81
Ring A, δ-lactol	1.00	0.16	—0.04
Δ^1—Double bond	0.12	0.13	0.70
1β-OH	—1.28	—0.44	0.49
2α-OH	—1.41	—1.40	—1.44
2β-OH	—1.21	—1.25	—1.50
Δ^3—Double bond	1.22	—0.79	0.14
3α-OH	—0.42	—0.64	—1.08
3β-OH	1.63	0.58	0.49
3β-OMe	0.13	—0.26	—1.09
3-Oxo	0.89	—0.42	—0.53
Δ^{16}—Double bond	—0.41	0.11	1.20
16β-Methyl	—1.28	—0.11	—0.05
16α-OH	—0.53	—1.18	—0.19
16α-Methyl	—0.32	0.29	1.10
13α-OH	0.05	—2.03	—0.35
12α-OH	—0.45	—2.46	—1.96
10α-Methyl, 4α-COOH	0.74	—0.84	—0.60
10α-COOH, 4α-COOH	0.97	—0.63	—0.28
2β-OH: 3β-OH interaction	—1.19	—1.56	—1.70
Multiple correlation *R*	0.939	0.920	0.876
Fisher's criterion $F_{k-1,N-k}$	11.301	9.332	5.620
Explained variance *EV*	0.793	0.756	0.632

On the whole, the good additivity of activity contributions speaks in favour of the primordial importance of the hormone-receptor contact for the display of growth promoting activity. High activity in all three bioassays is associated only with those C_{19} -gibberellins in which the set of substituents provides a high degree of topological

correspondence with the receptor. Among C_{20}-gibberellins high activity can be found when a compound either mimics these active C_{19}-gibberellins or rapidly produces them *in vivo*.

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Organic Chemistry

NOVEL ROUTES FOR SYNTHESIS OF PHENANTHRIDINE, BERBERINE AND APORPHINE ALKALOIDS

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Synthesis of the title alkaloids was accomplished through benzyne cyclisation and photo-initiated ring closures. Structure of fagaridine and some pentaoxygenated benzophenanthridine alkaloids has been confirmed through total synthesis. In contrast to deoxy substrates, photolysis of 1-(2′-halo-α-hydroxybenzyl)-1,2,3,4-tetrahydroisoquinolines furnished 7-hydorxy-aporphines smoothly. On irradiation, 1-ortho-toluyl-3,4-dihydroisoquinolines afford berberine alkaloids; the mechanism of this complex rearrangement has been investigated.

Keywords: Benzophenanthridine; Berberine; Aporphine; Benzyne; Photolysis

INTRODUCTION

SYNTHESIS of natural products exploiting new patterns of cyclization is of considerable chemical interest. In this context, we have explored a novel benzyne cyclisation and some photo-initiated ring closures for the synthesis of phenanthridine, berberine and aporphine alkaloids. Both types of reactions have been used essentially for forging an intramolecular aryl-aryl link in situations where earlier known procedures had been found unsatisfactory.

PHENANTHRIDINE ALKALOIDS

In the past decade, there has been renewed interest in developing effective routes to this group of alkaloids because some members e.g., nitidine and fagaronine, have shown promising lymphocytic activity. Our first effort was to develop an efficient approach to the phenanthridine nucleus which is also present in some synthetic compounds of pharmacological interest.

Benzyne Cyclisation

Perhaps the simplest way of building the phenanthridine ring system is through fusion of synthons A and B shown in Fig. 1. The azomethine link can be readily formed but difficulty arises in the subsequent ring closure. Since use of more conventional reactive intermediates, generated on one aromatic ring for attack on to the other, has been found to be inefficient, it was decided to explore benzynes for this purpose. Normally, aryl groups show little proclivity to attack benzyne in the desired fashion, but appendage of a negatively charged atom can confer sufficient nucleophilicity on the ortho and para positions:

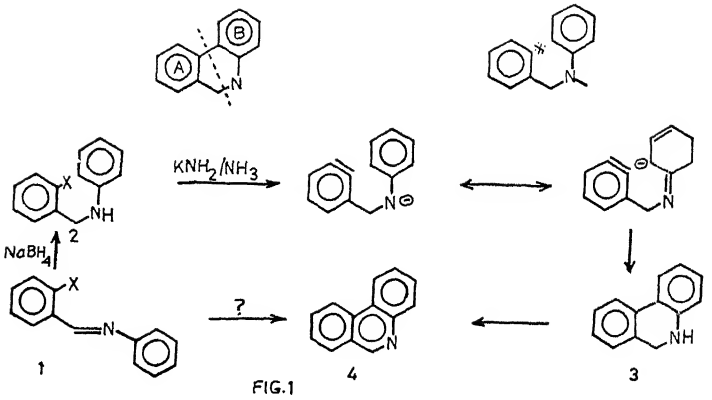

FIG.1

Treatment of the dihydroanil 2 with KNH_2/NH_3 indeed gave 3, in almost quantitative yield, which could be readily aromatised to phenanthridine. The cyclisation proceeded as well in the presence of a variety of substituents on either ring and constitutes a very convenient new synthesis. However, the sequence 1 to 4 entails two redundant steps—oxidation and reduction. It would be desirable to proceed directly from 1 to 4 which may seem impossible in view of the adverse trans geometry present in anils. Nevertheless, if rapid amide ion addition across the azomethine linkage occurs, the formed adduct would have anionic activation and can acquire the geometry requisite for cyclisation.

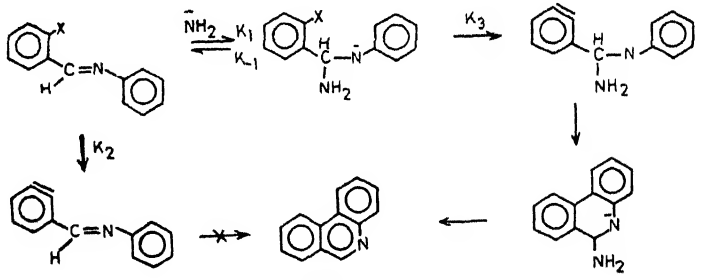

FIG.2

Experimentally direct reaction of *o*-chloro anil with KNH_2/NH_3 gave phenanthridine in excellent yield. Thus under the reaction conditions k_1 must be much larger than k_2 and k_{-1}. Fast addition of amide ions to anils was independently shown by an NMR study at −40°. Benzyne intermediacy in these cyclisations was demonstrated by ring closure of *meta*-halogenated substrates.

A number of condensed polynuclear heterocycles synthesised by the new phenanthridine route are shown. Some of these were elaborated to heterosteroids. It may be noticed that benzyne attack on hetero aromatic ring occurs efficiently and also naphthynes can be used as reactive intermediates. The diazapentacyclic system was obtained through a 'double benzyne cyclisation' of the dianil obtained from *o*-chlorobenzaldehyde and *p*-phenylenediamine. Of interest was the question

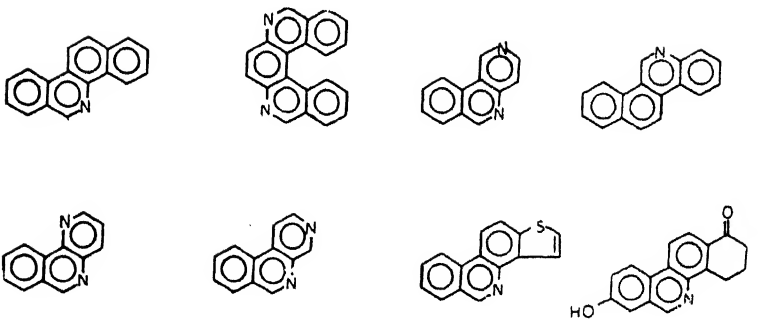

FIG 3

of pyridynes, specially for synthesis of the grass alkaloid perlolidine. It was found that both 2–3 and 3–4 pyridynes undergo this ring closure.

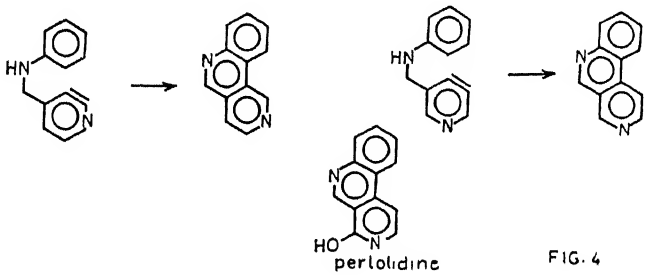

FIG. 4

The actual synthesis of perlolidine is shown in Fig. 5. It entails stepwise replacement of three bromine atoms by using reagents of increasing basic strength—a nice example of chemical selectivity.

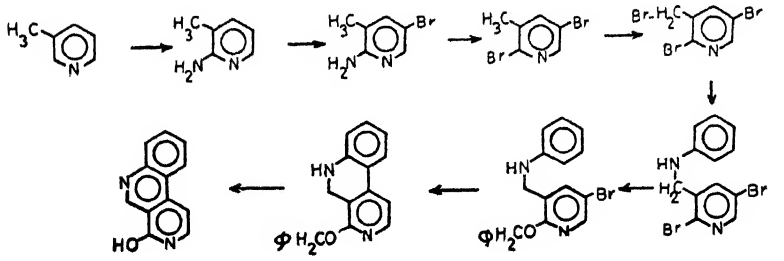

FIG.5

Turning to benzophenanthridines the benzyne cyclisation reaction was very successful for synthesis of 9,10-oxygenated alkaloids like chelerythrine.

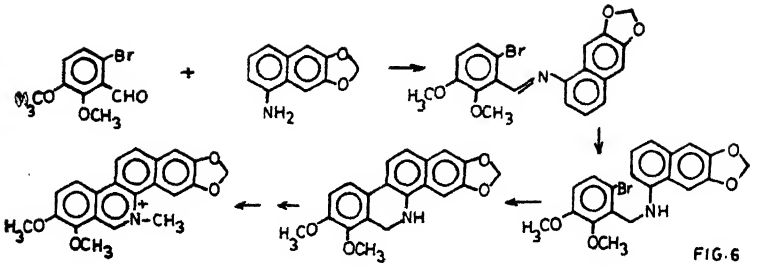

FIG. 6

Recently, a benzophenanthridine alkaloid has been isolated from Fagara Xanthoxyloides and named Fagaridine. On the basis of NMR and MS studies and conversion to chelerythrine, it was assigned the shown structure:

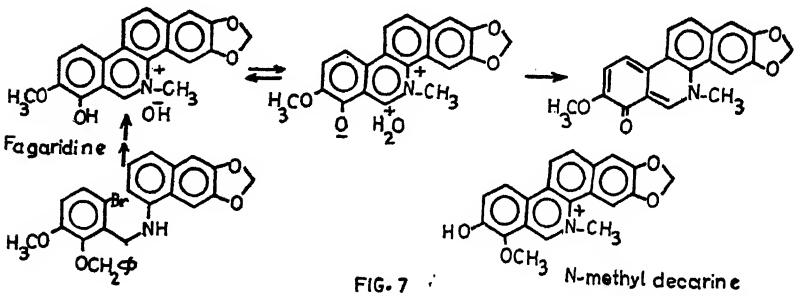

FIG. 7

On simple chemical grounds this assignment seems incompatible with the described pale colour of the alkaloid. Such a quaternary hydroxide should readily lose a molecule of water to give a highly conjugated quinoid structure exhibiting deep colour. This expectation was borne out by unambiguous synthesis, through benzyne cyclisation, of the compound corresponding to the proposed structure. Even a few crystals of this material impart intensive violet colour to a litre of solvent. We believe that the isolated alkaloid is a quaternary methio-salt of earlier known decarine.

Stermitz and coworkers have used the above procedure, which they term as Kessar benzyne cyclisation, to synthesise a number of benzo-phenanthridine compounds for anticancer screening and fagaranine was also synthesised for the first time. Nitidine and avicine were synthesized in our laboratory but the cyclisation yield in this class is modest as compared to that for chelerythrine. Therefore, it was decided to explore the following photochemical route for alkaloids carrying 10, 11-alkoxy substituents.

Photocyclisation of Orthohalogenated Anilides

Photocyclisation of orthohalogenated anilides was first studied by N. Kharasch and coworkers. We have extended it to synthesis of benzophenanthridines and have investigated the mode of the cyclisation. Two mechanisms, homolysis of the aryl halogen bond or electrocyclic ring closure, had been proposed earlier. Our studies

on correlation of the quantum yield with the nature of the halogen and solvent as well as Stern-Volmer kinetics of piperylene quenching seem to make both of these untenable. It is clear that two differentially quenchable excited states and aryl assisted homolysis are involved. Anyway, from the synthetic point of view the photocyclisation gave very satisfactory yields of nitidine and avicine.

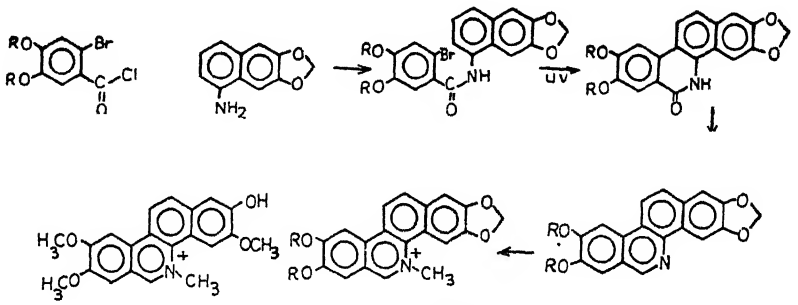

FIG.8

It was decided to extend this route for synthesis of pentaoxygenated benzophenanthridine alkaloids whose structure was matter of some controversy. Besides the oxygenation pattern first suggested by Slavik, two Japanese groups had proposed different placements of alkoxy substituents:

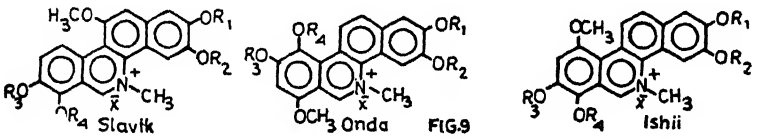

FIG.9

The route used for preparation of the substrates required for photocyclisation to Ishii structure is shown in Fig. 10. Unexpected difficulty was experienced in the amide formation step. The yield remained unsatisfactory even under drastic conditions. The reason may lie in crowding around the carboxyl group—a situation reminiscent of the problems in mesitoic acid esterification. Furthermore, irradiation of the bromoamide led to little cyclic product and the route was modified as shown:

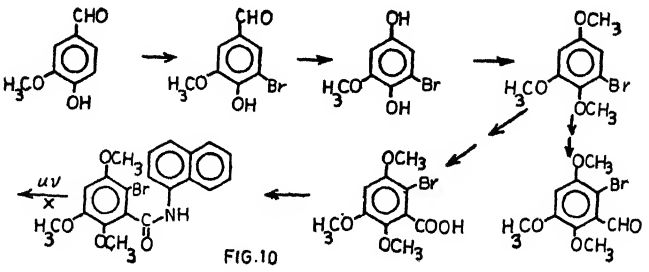

FIG.10

Photocylisation of Orthohalogenated Dihydroanils

The key step in the new approach is photocyclisation of the halogenated dihydroanils. This reaction proceeded in good yield and further elaboration afforded Chelilutine and sanguilutine, identical with authentic samples kindly provided by Professor Slavik.

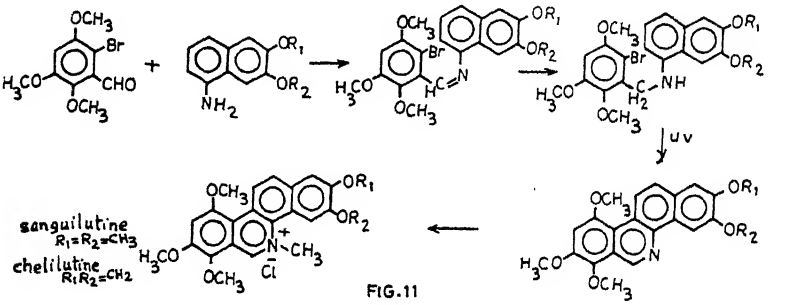

FIG.11

Recently, Professor Ninomiya has informed us that he has completed a synthesis, patterened on the above route, of the hexa-oxygenated alkaloid macarpine. It must be pointed that the photocyclisation of orthohalogenated dihydroanils looks very similar to that of corresponding benzanilides but is quite different in mechanism. Based on the work of Pac and Sakurai an electron transfer mechanism, shown in Fig. 12, may be proposed. However, the success of this photocyclisation is critically dependent on minimising the energy wasting back electron transfer process. We believe that immediately after electron transfer the nitrogen proton is lost which pushes the reaction in the forward direction. This proposal is substantiated by the observation that the cyclisation fails when an alkyl or acetyl substituent is present on the nitrogen atom.

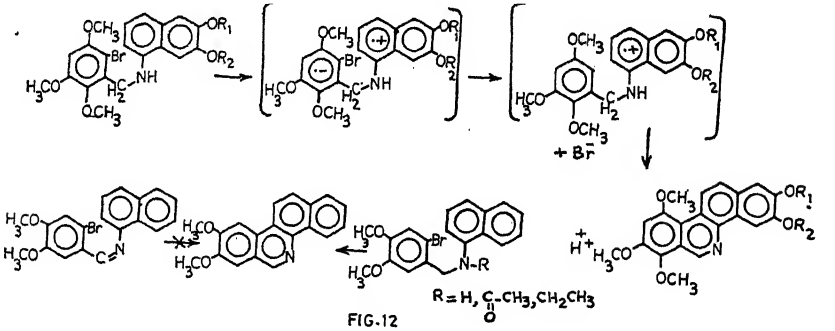

FIG.12

The critical role of electron transfer process in some photo reactions is discussed in the section on aporphine alkaloids also. However, before taking that up it would be appropriate to comment on the merits and deficiencies of the three routes developed for synthesis of benzophenanthridine alkaloids. Benzyne cyclisation has the advantage of large scale (upto 10gm cyclisation batches can be handled easily) and

is the method of choice for 9, 10-oxygenated alkaloids. However, for 10,11-oxygenated alkaloids, the yield by this procedure is modest and halogenated amide photocyclisation is advantageous. For alkaloids having an alkoxy group in position 12 benzyne cyclisation is inapplicable while the amide photocyclisation approach turned out to be unrewarding. However, electron-transfer mediated photocyclisation proved very useful for synthesis of this group of alkaloids.

Aporphine Alkaloids

Aporphines constitute a big group of isoquinoline alkaloids some of which show valuable pharmacological activity. A large number of these have been synthesised, primarily through the Pschorr reaction of 1–(2′-aminobenzyl)–1, 2, 3, 4-tetrahydroisoquinolines. The yields in this cyclisation are often poor which has prompted the search for alternate procedures. We were specially interested in a route suitable for the synthesis of 7-hydroxy aporphines, particularly the diastereomer in which C-6a and C-7 hydrogens are trans to each other. A number of alkaloids of this geometry had been isolated but no synthesis has been reported.

Photocyclisation of 1-(2′-Halobenzyl)-1, 2, 3, 4-Tetrahydroisoquinolines

Quite a few reports on photolysis of 1–(2′-halobenzyl)-tetrahydroisoquinolines to aporphines have appeared in literature. A comprehensive analysis of this reaction suggests that electron transfer processes and conformational effects play a vital role in it. Roughly, the cyclisation may be divided into three categories:

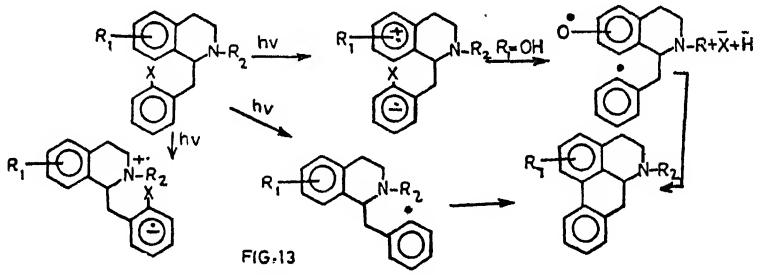

FIG. 13

(i) Simple bases, in which $R_2 = H$ or CH_3 and $R_1 \neq OH$, on irradiation afford complex mixtures containing hardly any aporphines. Perhaps electron transfer from the nitrogen to the aryl moiety, as suggested by Kupchan, occurs and the formed ammonium radical ions fragment to give side products;

(ii) This pathway may be avoided by carrying out the photolysis in an acidic medium or by using substrates in which R_2 is an electron withdrawing substituent, like an acetyl group. However, even when the availability of the electron pair on the nitrogen atom is decreased, the aporphine yields in the photocyclisation are poor, sometimes less than 5 per cent. In such cases simple aryl-halogen bond homolysis probably occurs but the radical may be generated in, or may acquire, a conformation in which the aryl moieties are too far apart for ring closure to occur. Thus prior to rotation, other radical reactions, like hydrogen abstraction and dimerisation, may intercede to reduce the cyclisation yields;

(iii) In the third class may be placed substrates in which R_1 = OH and the yields are good. If the irradiation is carried out in a basic medium the electron transfer may occur from the phenoxide (in a non-basic medium from phenol accompanied by a rapid proton loss) to eventually give a diradical in which the two aryl groups are close to each other. Anyway, combination of radicals is expected to be a favoured process and efficient photocyclisation may thus be rationalised.

On basis of the above analysis, it seemed that if somehow the deleterious electron transfer from the nitrogen atom and the unfavourable geometry for radical cyclisation could be avoided, reasonable yields may be obtained in photocyclisation to non-phenolic aporphines also. In case of 1-(2'-halo-α-hydroxybenzyl)-1, 2, 3, 4-tetrahydroisoquinoline both these requirements may be met, if intramolecular hydrogen bonding (Fig. 14) occurs. The nitrogen and aryl group will be too far apart for electron transfer and the two aryl moieties would remain close to each other. This could result in an efficient photocyclisation to give the 7-hydroxyaporphines which were of special interest to us for reasons discussed earlier.

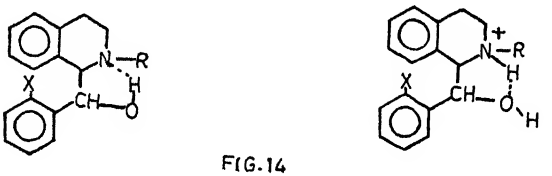

FIG. 14

Spectroscopic data obtained on 1-(2'-halo-α-hydroxybenzyl)-1, 2, 3, 4-tetrahydroisoquinolines suggest presence of the conformations favourable for cyclisation. Specially in the NMR spectrum, the C-8 aromatic proton appears higher field in these than in 1-benzyltetrahydroisoquinolines devoid of α-hydroxy function. This suggests a greater population of the conformation in which C-8 proton lies near and above the plane of the appended aryl ring. Anyway, irradiation of 2'-halo-α-hydroxy compounds in aqueous HCl gave cyclisation yields in the range of 30 per cent. More dramatic results were obtained in photocyclisation of these substrates in cyclohexane. The 7-hydroxyaporphines were again obtained in good yield (40 per cent); while in absence of the α-hydroxy group photocyclisation under these conditions is known to fail completely. Irrespective of the validity of the rationalisation of this approach, it gave the target molecules readily and synthesis of oliveroline and oliveridine could be accomplished.

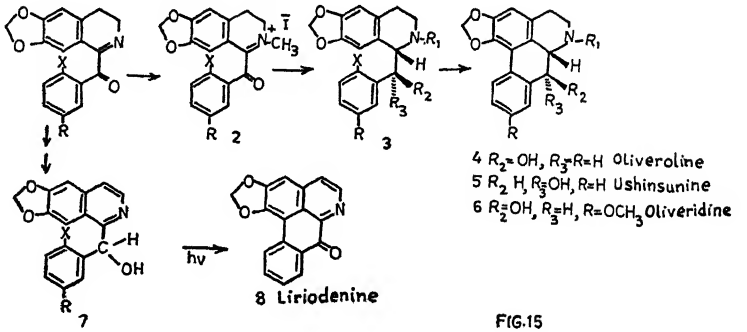

FIG. 15

One of the reasons why these alkaloids had not been synthesised earlier, in contrast to the *cis* analogues, is perhaps the unfavourable stereochemistry encountered in reduction of keto-imine substrates. Reduction under various conditions affords the undesired 1R, αS diastereomer almost exclusively. We found that sodium borohydride reduction of the corresponding quaternary compounds gives substantial quantities of the 1R, αR diastereomer. The composition of the obtained mixture can be monitored by NMR spectroscopy and it was observed that larger the steric bulk of nitrogen substituent more is the proportion of the 1R, αR diastereomer. Another aspect needing comment is the fact that photocyclisation of 1R, αR substrates proceeds more efficiently. Thus an equimolar mixture of the diastereomers on short irradiation gives the aporphine alkaloid with the *trans* stereochemistry almost exclusively. The reasons for this selectivity are, however, not clear.

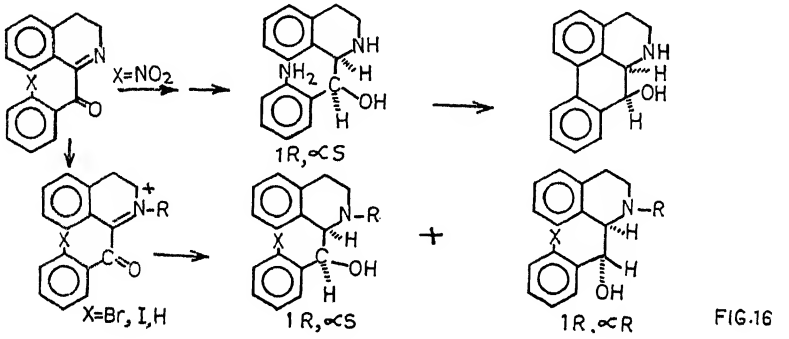

FIG.16

Benzyne Cyclisation

Success in benzyne cyclisation involving nitranion activation of the attached aromatic ring, prompted us to explore oxyanion activated cyclisations. The substrates selected for this purpose are shown in Fig. 17. It is clear that three nucleophilic sites are available for attack by the generated benzyne. In case of path (*a*) aporphine alkaloids could result while (*b*) would lead to spiroquinone structures and (*c*) would furnish dibenzopyrrocoline alkaloids. In practice, all the three products were formed; the first and the last type alkaloids were obtained in yields ranging from 20–30 per cent whereas amurine could be obtained only in 2 per cent yield. Simultaneously with our work Kametani and coworkers also investigated this cyclisation with similar results.

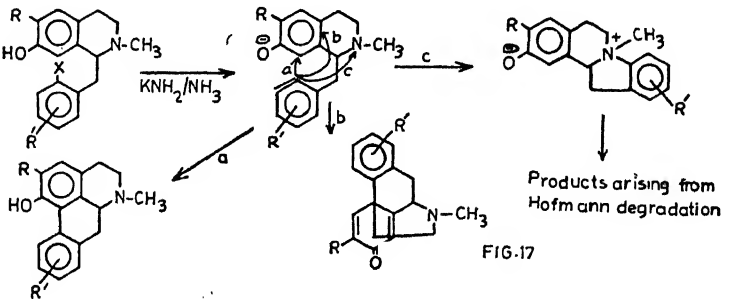

FIG.17

Berberine Alkaloids

Benzyne Cyclisation

It was envisaged to exploit nucleophilicity of enamine carbon atoms to bring about a benzyne cyclisation leading to berberine skeleton.

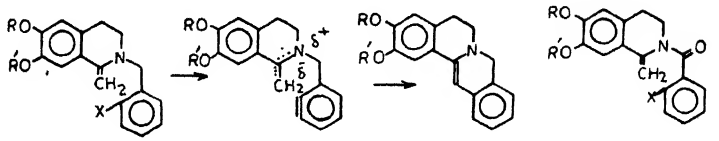

FIG. 18

Treatment of the shown enamine with KNH_2/NH_3 furnished only amination products. However, when R was hydrogen and oxy anion, generated in the basic medium, was available for activation of the enamine carbon the desired cyclisation was observed. The requirement of a suitably placed phenolic function severly limits the usefulness of this approach. The Ninomiya enamide photocyclisation is a far more versatile procedure. Incidentally, benzyne cyclisation with corresponding enamides was explored by Kametani's group and yields in the range of 10–20 per cent were obtained.

Photocyclisation of 1-Toluyl-3, 4-Dihydroisoquinolines

Photolysis of 1-toluyl-3, 4-dihydroisoquinolines has been investigated in detail not only as a route to tetrahydroberberine alkaloids but also because of our basic interest in imine photochemistry. This functionality lies, at least on electronegativity basis, between the $\rangle C \quad O$ and $\rangle C = C \langle$ groups but fails to exhibit photo reactions characteristic of either. Energy wasting *trans-cis* isomerisation in the excited state has often been held responsible for this photo-inactivity. However, incorporation of the $\rangle C = N-$ bond in a five or six membered ring does not confer hydrogen abstraction activity. The search for photo reactions of imines has led to investigation of modified functionality and we have looked at α-ketoimines in this context. The actual substrate used and its synthesis are shown in Fig. 19.

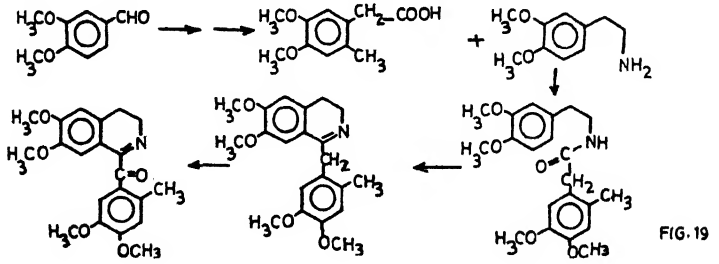

FIG. 19

This particular keto-imine was chosen because the $\rangle C \quad N \langle$ bond is incorporated in a ring and it is juxtaposed with an easily abstractable benzylic hydrogen. Further,

this reactant could lead to interesting alkaloid structures. If hydrogen abstracton by the $\rangle C = N\langle$ bond occurs in the excited state (path *a*), the formed diradical can collapse to give the spiro benzylisoquinoline alkaloids. However, this product may undergo a known photo-initiated α-cleavage and lead to, after internal hydrogen abstraction by the acyl radical and ring closure, berberine type of alkaloids. The

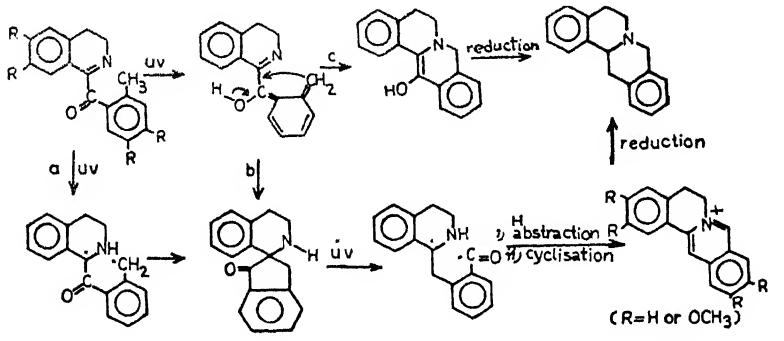

FIG. 20

spiro compound could also arise by carbonyl hydrogen abstraction followed by hydrogen migration and ring closure (path *b*). In a third possibility, the formed enol could undergo electrocyclic ring closure to a tetracyclic structure (path *c*) suitable for elaboration to berberine alkaloids.

Irradiation (8 hrs) of 1 (with appropriate alkoxy groups) in methanol using a pyrex filter gave 2, in about 50 percent yield, which could readily be reduced to xylopinine. Alkaloids substituted in C-13 position could be similarly obtained by starting with ethyl analogue of 1. For extending this synthesis to more abundant 9, 10-oxygenated alkaloids, the required substrate was secured through a photo rearrangement of a phenoxy acid. It was elaborated to the keto-imine irradiation of which afforded

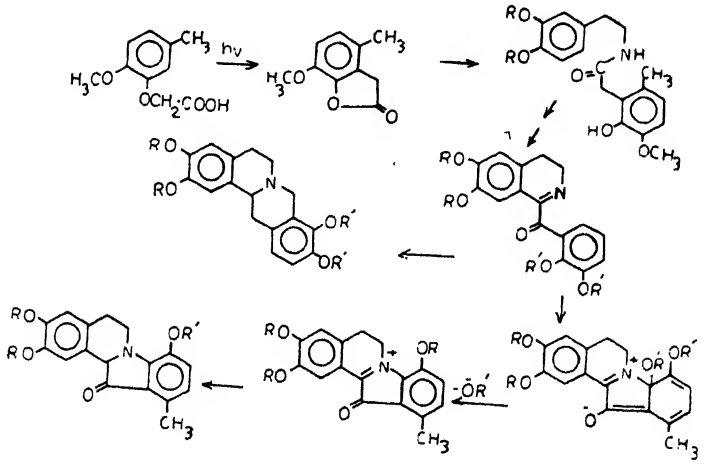

FIG. 21

little tetrahydro palmitine but another product tentatively assigned a keto indole structure was obtained. Its formation is rationalised on the basis of an electrocyclic ring closure followed by loss of methoxide ion. However, the corresponding methylene dioxy keto-imine (R_1, R_2 = O-CH_2-O) on irradiation did give sinactine in poor yield.

Finally, some mechanistic aspects of this novel photo reaction have been investigated. A distinction between paths *a*, *b* on one hand and *c* on the other (Fig. 20) can be made. It may be noted that in the latter path the benzylic carbon gets attached to the nitrogen atom whereas in the spiro ketone route the benzylic carbon gets linked to C-1 from which the ketonic carbon is detached and is joined to the nitrogen atom. To distinguish between the two possibilities the required unsymmetrically substituted substrate was obtained by a dihydro berberine photo degradation recently reported by Shamma and coworkers:

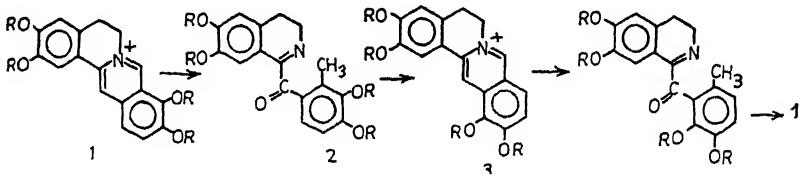

FIG. 22

Irradiation of the obtained keto-imine 1 furnished a berberine which was different from the starting material and could be assigned structure 3 on basis of mass and NMR data. Thus path *c* (Fig. 20) can be ruled out. The sequence 1–3 amounts to striking switching of ring D substituents of a berberine alkaloid.

Having established that this novel photo-reaction proceeds through a spiro ketone intermediate, another aspect of the mechanism was looked into. Subsequent to ∝-cleavage the acyl radical can abstract a hydrogen from the nitrogen atom or from the benzylic carbon. In each case, the berberinium salt can be formed on ring closure. There is no *a priori* reason to consider one mode preferable to the other

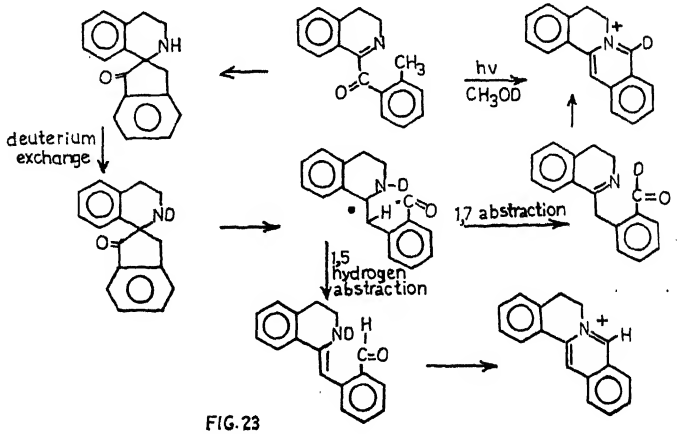

FIG. 23

(7 membered ring versus 5 membered ring). When the keto-imine irradiation was carried out in CH_3OD a deuterium atom got incorporated in position C-8. Thus the deuterium introduced on the nitrogen atom after exchange gets preferentially abstracted by the acyl radical.

ACKNOWLEDGEMENTS

In the end I wish to express my deep gratitude to my coworkers specially Dr Manmohan Singh, Dr Prasanna Balakrishnan, Dr Shuchita Narula, Dr Paramjit Singh, Dr Y. P. Gupta, Dr S. S. Gandhi, Dr Tej Vir Singh, Mr K. K. Sawal, Mr Taj Mohammad and Mr Ashok Nanda.

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Bioorganic Chemistry

DNA-DEPENDENT RNA POLYMERASE FROM *E. coli*: STRUCTURE-FUNCTIONAL STUDIES

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(*Received 15 October 1981*)

In order to determine the primary structure of large β- and β′-subunits of *E. coli* RNA polymerase the protein fragmentation and sequencing procedure were combined with sequencing of the corresponding structural genes. In all 4714 base pairs of the rpoBC operon were sequenced including the entire structural gene of the β-subunit and an initial segment (528 base pairs) of the β′ structural gene. The β-subunit appeared to consist of 1342 amino acid residues from which molecular weight was calculated to be 150618.6 daltons.

Spatial organization of RNA polymerase was studied with the aid of chemical modification and limited proteolysis. Topography of cystein and tyrosine residues were elucidated in the case of the α-subunit. Accessibility of different areas of the β-subunit polypetide chain to limited tryptic hydrolysis was examined.

Chemical and photochemical affinity modifications were used to find out subunits of RNA polymerase responsible for binding of DNA template, substrates and RNA product. Photoinduced covalent binding of RNA polymerase to DNA fragments containing 5-bromodeoxyuridine showed that in binary complex along with β- and β′-subunits σ-factor contacts with DNA.

We have developed an approach enabling us to detect contacts of RNA polymerase with 5′- and 3′-ends of growing RNA chain on different stage of transcription. On early stages mainly σ-subunit contacts with 3′-end, ββ′ and σ with 5′-end of the nascent RNA chain. On the later stages only β and β′ contacts with 5′-end of RNA. Hence, the role of the σ-subunit is not limited by recognition of the promoter, it may also directly participate in RNA synthesis initiation.

Keywords: RNA Polymerase; Amino Acid Sequence; Nucleotide Sequence; Binding Sites

INTRODUCTION

TRANSCRIPTION of genetic information in bacterial cells is mediated by DNA-dependent RNA polymerase. As yet little is known of the mechanism of RNA polymerase action, and of the role of its individual components in the transcription process. Progress in its study is hindered by lack of information about the enzyme primary and spatial structures. This has prompted us to initiate an investigation into the primary structures of the *E. coli* RNA polymerase subunits.

Earlier we had established the amino acid sequence of the α-subunit of *E. coli* DNA-dependent RNA polymerase resorting solely to the ordinary methods of protein chemistry (Ovichinnikov *et al.*, 1977). According to the primary structure

the polypeptide chain of the α - subunit has molecular weight 36512 and consists of 329 amino acid residues. In the case of the β- and β′-subunits with their much higher molecular weights (~155.000 and ~ 165.000, respectively), such an approach could no longer suffice.

One of the approaches to the structure determination of large protein molecules is their initial cleavage into a small number of fragments, which then can be analyzed by conventional methods. The search for the conditions of limited proteolysis of the β - and β′-subunits was undertaken. Considerable obstacles were encountered in the course of these studies owing to the fact that the RNA polymerase subunits are not the native proteins. However, the conditions for limited tryptic proteolysis of the β - subunit were found. These are an enzyme/substrate ratio of 1:500, temperature 0 °C, 4hr (Marchenko *et al.*, 1980). Herein forms an optimal set of large fragments (mol. wt 62.000, 52.000, 37.000, 24.000 and 10.000). Initial separation of the resultant hydrolysate was carried out by chromatography on Sephadex G-100 in 6 M guanidine hydrochloride. This yielded 10 fractions. Their analysis by polyacrylamide gel electrophoresis showed that all large fragments mentioned above are in the first three fractions, while the rest contain about 95 smaller peptides. 53 low molecular weight peptides were isolated from the hydrolysate. They consist of approximately 400 amino acid residues. Their sequencing was very useful for a further structure investigation (Lipkin *et al.*, 1980). Isolation of the high molecular peptides proved difficult because of both the little hydrolytic specificity and the low yield of most products. So we could not use limited proteolysis as the main procedure for β-sequencing.

The progress in DNA sequencing methods allowed to realize the possibility of using the genetic code to obtain information on the primary protein structure from the nucleotide sequences. However, there are many pitfalls in the way, requiring considerable caution to avoid possible sources of error.

In the first place the mRNA can undergo processing, leading to erroneous deduction of the protein structure. This holds particularly for eukaryotic cells, wherein "splicing" has been noted. Secondly, the protein itself can be processed. Thirdly, it is often difficult to recognize in the overall DNA structure the beginning of a structural gene. Moreover, one has to bear in mind that a single error (deletion or insertion) in the DNA sequence could lead to a completely erroneous amino acid sequence of the protein.

Thus, primary structure determination of DNA cannot serve as a substitute for the direct sequencing of the protein. In view of this, we decided to utilize the methods of both protein and nucleotide chemistries, performing the parallel sequencing of the structural genes rpoB (β-subunit) and rpoC (β′-subunit) and of the corresponding proteins. Knowledge of the nucleotide sequence of the pertinent DNA segments would permit aligning of the peptide fragments from the protein analysis into an uninterrupted polypeptide chain. Such an approach provides the key to the most complicated problem in the primary structure analysis of high molecular proteins.

In Fig. 1, restriction endonucleases cleavage map of *E. coli* DNA region containing the structural genes of the β and β′ subunits of the RNA polymerase (rpoB and rpoC correspondingly) is given. We determined the total sequence of the EcoRI-C (Ovchinnikov *et al.*, 1980 *a*) EcoRI-F (Monastyrskaya *et al.*, 1980 *a*) and

EcoRI-A—Hind III fragments and partial sequence of the EcoRI-G fragment carrying the beginning of the rpoB gene (Monastyrskaya *et al.*, 1980 *b*). These fragments were obtained from DNA of λ_{rif}^{d} 47 and λ_{rif}^{d} 18 transducing phages, containing the *E. coli* rpoBC operon (Kirschlaum & Konrad, 1973; and Mindlin *et al.*, 1976), or corresponding plasmids by EcoRI restriction endonuclease digestion. In the case of EcoRI-A-Hind III fragment EcoRI and Hind III digestions were used. The fragments were consecutively digested with one of the restriction endonucleases (Sau 3AI, Hinf I, Hpa II and Taq I) cleaving the DNA into relatively small blocks. The resulting subfragments were phosphorylated by means of | γ^{-32} P | - ATP and phage T4 polynucleotide kinase and the mixture was separated by electrophoresis on polyacrylamide gel. As a rule, both complementary chains obtained after denaturation of each subfragment and separation were analyzed. Their sequencing was performed by a modified Maxam-Gilbert procedure.

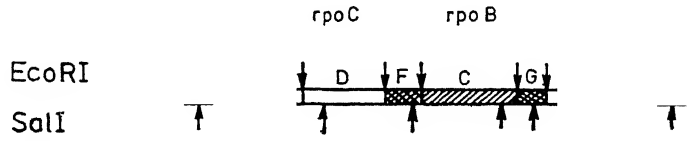

FIG. 1. EcoRI and SalI restriction cleavage map of the *E. coli* DNA region including the structural genes (rpoB and rpoC) of the β and β′ RNA polymerase subunits.

As another method of the β-subunit polypeptide chain splitting, the digestion with *Staphylococcus aureus* protease was chosen. Initial fragmentation of the hydrolysate was performed by gel filtration on biogel P-4. This yielded four fractions. Subsequent separation of the peptides was achieved by chromatography on the cation exchanger AG-50Wx4 and paper chromatography. Fraction I contained mixture of the largest peptides. To facilitate the separation and analysis of the peptides in this fraction the mixture was additionally digested with chymotrypsin. Altogether 3 peptides were isolated from fraction IV, 73 from fraction III, 48 from fraction II and 60 from fraction I.

In order to obtain the missing fragments, the exhaustive tryptic digestion of the β-subunit was carried out after modification of the lysine residues with citraconic anhydride. The tryptic peptides were separated according to the same scheme as that used for staphylococcal peptides. After removal of the citraconic protection the high molecular weight peptides were subjected to additional tryptic cleavage at the lysine residues. In order to determine the primary structure of the β′-subunit its cyanogen bromide cleavage was shown. For separation of the resulting peptides we used gel filtration, paper and thin layer chromatographies, electrophoresis in acetate cellulose slabs and butanol extraction of hydrophobic peptides. The tryptic digestion of the β′-subunit was also carried out after modification of the lysine residues with citraconic anhydride. The amino acid sequence of the peptide fragments determined up to now covers more than 80 per cent of the total β-subunit peptide chain and about 30 per cent of the β′-subunit polypeptide chain.

During this investigation the comparison of the nucleotide and amino acid sequence was carried out continually. The search for similarities between the amino

acid sequences of the peptides and the nucleotide sequences of the corresponding DNA fragments was carried out by means of a computer. A conjunction of the methods of protein and nucleotide chemistry for the combined structural investigation of a protein and DNA sharply accelerated and considerably simplified the solution of both problems and enhanced the reliability of the structural analysis. As a result, the nucleotide sequences of the two segments of the rpoBC operon (4714 and 2306 base pairs) embracing the entire rpoB gene, the initial and terminal parts of the rpoC gene and the intercistronic region, together with the total amino acid sequence of the β-subunit, comprising 1342 residues (Ovchinnikov *et al.*, 1980 *b*, 1981 and the N- and C-terminal sequences of the β′-subunit (176 and 421 residues) were determined (Figs. 2, 3).

Spatial organization of RNA polymerase was studied with the aid of chemical modification and limited proteolysis. A topographic study of the RNA polymerase subunits was made by iodinating the tyrosine residues (Lipkin *et al.*, 1979) and carboxymethylating the cysteine residues of the core-enzyme and of the individual subunits. It was found that only a limited number of tyrosine and cysteine residues are exposed on the RNA polymerase surface. This number is significantly augmented when each subunit is modified separately, evidence of the considerable area of mutual contact between the subunit components of core-enzyme. Knowledge of the primary structure of the α-subunit made possible discrimination between tyrosine and cysteine residues found on the core-enzyme surface (Tyr^{68}, Tyr^{277}, Cys^{269}), in the region of contact with the other subunits (Tyr^{153}, Tyr^{185}) and buried within the globule (Tyr^{177}, Cys^{54}, Cys^{131}, Cys^{176}). The sequencing of the peptides isolated from β-subunit limited tryptic hydrolysate enabled to find regions of polypeptide chain easily accessible for the proteolytic digesting. Two regions in the middle part of the β-subunit polypeptide chain (amino acid residues 528–548 and 678–758) and C-terminal part of the molecule (residues 1105–1342) are evidently on the surface of the protein and possibly represent flexible connections between stable domains.

Chemical and photochemical affinity modifications were used to find subunits of the RNA polymerase responsible for binding the DNA template, substrates and RNA product.

It is known that short thymidilate polynucleotides serve as templates for RNA polymerase. Decanucleotide 5′-^{32}P-d (T_5-BrU-T_4) containing photoreactive residue of 5-bromouracil forms a stable covalent complex with RNA polymerase subunits under ultraviolet irradiation. The distribution of the radioactive label among the subunits depends on the type of enzyme (holo or core) and the presence of ATP. The irradiation of the binary holoenzyme-de-canucleotide complex has led to preferential labelling of the σ-subunit along with β- and β′-subunits. In the presence of ATP (transcribing complex) β′ was labelled exclusively (Ovchinnikov *et al.*, 1979).

We have developed an approach enabling us to detect contacts of RNA polymerase with 5′- and 3′-ends of the growing RNA chain at different stages of transcription. To identify the enzyme contacts with the 5′-end of the nascent RNA chain we used a one promoter-containing fragment of a $\lambda_{imm}434$ DNA as a template,

```
1-81      TTC CGG TCA ACA AAA TAG TGT TGC ACA AAC TGT CCG CTC AAT GGA CAG ATG GGT CGA CTT GTC AGC GAG CTG AGG AAC CCT

82-162    ATG GTT TAC TCC TAT ACC GAG AAA AAA CGT ATT CGT AAG GAT TTT GGT AAA CGT CCA CAA GTT CTG GAT GTA CCT TAT CTC
1-27      Met-Val-Tyr-Ser-Tyr-Thr-Glu-Lys-Lys-Arg-Ile-Arg-Lys-Asp-Phe-Gly-Lys-Arg-Pro-Gln-Val-Leu-Asp-Val-Pro-Tyr-Leu-

163-243   CTT TCT ATC CAG CTT GAC TCG TTT CAG AAA TTT ATC GAG CAA GAT CCT GAA GGG CAG TAT GGT CTG GAA GCT GCT TTC CGT
28-54     Leu-Ser-Ile-Gln-Leu-Asp-Ser-Phe-Gln-Lys-Phe-Ile-Glu-Gln-Asp-Pro-Glu-Gly-Gln-Tyr-Gly-Leu-Glu-Ala-Ala-Phe-Arg-

244-324   TCC GTA TTC CCG ATT CAG AGC TAC AGC GGT AAT TCC GAG CTG CAA TAC GTC AGC TAC CGC CTT GGC GAA CCG GTG TTT GAC
55-81     Ser-Val-Phe-Pro-Ile-Gln-Ser-Tyr-Ser-Gly-Asn-Ser-Glu-Leu-Gln-Tyr-Val-Ser-Tyr-Arg-Leu-Gly-Glu-Pro-Val-Phe-Asp-

325-405   GTC CAG GAA TGT CAA ATC CGT GGC GTG ACC TAT TCC GCA CCG CTG CGC GTT AAA CTG CGT CTG GTG ATC TAT GAG CGC GAA
82-108    Val-Gln-Glu-Cys-Gln-Ile-Arg-Gly-Val-Thr-Tyr-Ser-Ala-Pro-Leu-Arg-Val-Lys-Leu-Arg-Leu-Val-Ile-Tyr-Glu-Arg-Glu-

406-486   GCG CCG GAA GGC ACC GTA AAA GAC ATT AAA GAA CAA GAA GTC TAC ATG GGC GAA ATT CCG CTC ATG ACA GAC AAC GGT ACC
109-135   Ala-Pro-Glu-Gly-Thr-Val-Lys-Asp-Ile-Lys-Glu-Gln-Glu-Val-Tyr-Met-Gly-Glu-Ile-Pro-Leu-Met-Thr-Asp-Asn-Gly-Thr-

487-567   TTT GTT ATC AAC GGT ACT GAG CGT GTT ATC GTT TCC CAG CTG CAC CGT AGT CCG GGC GTC TTC TTT GAC TCC GAC AAA GGT
136-162   Phe-Val-Ile-Asn-Gly-Thr-Glu-Arg-Val-Ile-Val-Ser-Gln-Leu-His-Arg-Ser-Pro-Gly-Val-Phe-Phe-Asp-Ser-Asp-Lys-Gly-

568-648   AAA ACC CAC TCT TCG GGT AAA GTG CTG TAT AAC GCG CGT ATC ATC CCT TAC CGT GGT TCC TGG CTG GAC TTC GAA TTC GAT
163-189   Lys-Thr-His-Ser-Ser-Gly-Lys-Val-Leu-Tyr-Asn-Ala-Arg-Ile-Ile-Pro-Tyr-Arg-Gly-Ser-Trp-Leu-Asp-Phe-Glu-Phe-Asp-

649-729   CCG AAG GAC AAC CTG TTC GTA CGT ATC GAC CGT CGC CGT AAA CTG CCT GCG ACC ATC ATT CTG CGC GCC CTG AAC TAC ACC
190-216   Pro-Lys-Asp-Asn-Leu-Phe-Val-Arg-Ile-Asp-Arg-Arg-Arg-Lys-Leu-Pro-Ala-Thr-Ile-Ile-Leu-Arg-Ala-Leu-Asn-Tyr-Thr-

730-810   ACA GAG CAG ATC CTC GAC CTG TTC TTT GAA AAA GTT ATC TTT GAA ATC CGT GAT AAC AAG CTG CAG ATG GAA CTG GTG CCG
217-243   Thr-Glu-Gln-Ile-Leu-Asp-Leu-Phe-Phe-Glu-Lys-Val-Ile-Phe-Glu-Ile-Arg-Asp-Asn-Lys-Leu-Gln-Met-Glu-Leu-Val-Pro-

811-891   GAA CGC CTG CGT GGT GAA ACC GCA TCT TTT GAC ATC GAA GCT AAC GGT AAA GTG TAC GTA GAA AAA GGC CGC CGT ATC ACT
244-270   Glu-Arg-Leu-Arg-Gly-Glu-Thr-Ala-Ser-Phe-Asp-Ile-Glu-Ala-Asn-Gly-Lys-Val-Tyr-Val-Glu-Lys-Gly-Arg-Arg-Ile-Thr-

892-972   GCG CGC CAC ATT CGC CAG CTG GAA AAA GAC GAC GTC AAA CTG ATC GAA GTC CCG GTT GAG TAC ATC GCA GGT AAA GTG GTT
271-297   Ala-Arg-His-Ile-Arg-Gln-Leu-Glu-Lys-Asp-Asp-Val-Lys-Leu-Ile-Glu-Val-Pro-Val-Glu-Tyr-Ile-Ala-Gly-Lys-Val-Val-

973-1053  GCT AAA GAC TAT ATT GAT GAG TCT ACC GGC GAG CTG ATC TGC GCA GCG AAC ATG GAG CTG AGC CTG GAT CTG CTG GCT AAG
298-324   Ala-Lys-Asp-Tyr-Ile-Asp-Glu-Ser-Thr-Gly-Glu-Leu-Ile-Cys-Ala-Ala-Asn-Met-Glu-Leu-Ser-Leu-Asp-Leu-Leu-Ala-Lys-

1054-1134 CTG AGC CAG TCT GGT CAC AAG CGT ATC GAA ACG CTG TTC ACC AAC GAT CTG GAT CAC GGC CCA TAT ATC TCT GAA ACC TTA
325-351   Leu-Ser-Gln-Ser-Gly-His-Lys-Arg-Ile-Glu-Thr-Leu-Phe-Thr-Asn-Asp-Leu-Asp-His-Gly-Pro-Tyr-Ile-Ser-Glu-Thr-Leu-

1135-1215 CGT GTC GAC CCA ACT AAC GAC CGT CTG AGC GCA CTG GTA GAA ATC TAC CGC ATG ATG CGC CCT GGC GAG CCG CCG ACT CGT
352-378   Arg-Val-Asp-Pro-Thr-Asn-Asp-Arg-Leu-Ser-Ala-Leu-Val-Glu-Ile-Tyr-Arg-Met-Met-Arg-Pro-Gly-Glu-Pro-Pro-Thr-Arg-

1216-1296 GAA GCA GCT GAA AGC CTG TTC GAG AAC CTG TTC TTC TCC GAA GAC CGT TAT GAC TTG TCT GCG GTT GGT CGT ATG AAG TTC
379-405   Glu-Ala-Ala-Glu-Ser-Leu-Phe-Glu-Asn-Leu-Phe-Phe-Ser-Glu-Asp-Arg-Tyr-Asp-Leu-Ser-Ala-Val-Gly-Arg-Met-Lys-Phe-

1297-1377 AAC CGT TCT CTG CTG CGC GAA GAA ATC GAA GGT TCC GGT ATC CTG AGC AAA GAC GAC ATC ATT GAT GTT ATG AAA AAG CTC
406-432   Asn-Arg-Ser-Leu-Leu-Arg-Glu-Glu-Ile-Glu-Gly-Ser-Gly-Ile-Leu-Ser-Lys-Asp-Asp-Ile-Ile-Asp-Val-Met-Lys-Lys-Leu-

1378-1458 ATC GAT ATC CGT AAC GGT AAA GGC GAA GTC GAT GAT ATC GAC CAC CTC GGC AAC CGT CGT ATC CGT TCC GTT GGC GAA ATG
433-459   Ile-Asp-Ile-Arg-Asn-Gly-Lys-Gly-Glu-Val-Asp-Asp-Ile-Asp-His-Leu-Gly-Asn-Arg-Arg-Ile-Arg-Ser-Val-Gly-Glu-Met-

1459-1539 GCG GAA AAC CAG TTC CGC GTT GGC CTG GTA CGT GTA GAG CGT GCG GTG AAA GAG CGT CTG TCT CTG GGC GAT CTG GAT ACC
460-486   Ala-Glu-Asn-Gln-Phe-Arg-Val-Gly-Leu-Val-Arg-Val-Glu-Arg-Ala-Val-Lys-Glu-Arg-Leu-Ser-Leu-Gly-Asp-Leu-Asp-Thr-

1540-1620 CTG ATG CCA CAG GAT ATG ATC AAC GCC AAG CCG ATT TCC GCA GCA GTG AAA GAG TTC TTC GGT TCC AGC CAG CTG TCT CAG
487-513   Leu-Met-Pro-Gln-Asp-Met-Ile-Asn-Ala-Lys-Pro-Ile-Ser-Ala-Ala-Val-Lys-Glu-Phe-Phe-Gly-Ser-Ser-Gln-Leu-Ser-Gln-

1621-1701 TTT ATG GTC CAG AAC AAC CCG CTG TCT GAG ATT ACG CAC AAA CGT CGT ATC TCC GCA CTC GGC CCA GGC GGT CTG ACC CGT
514-540   Phe-Met-Val-Gln-Asn-Asn-Pro-Leu-Ser-Glu-Ile-Thr-His-Lys-Arg-Arg-Ile-Ser-Ala-Leu-Gly-Pro-Gly-Gly-Leu-Thr-Arg-

1702-1782 GAA CGT GCA GGC TTC GAA GTT CGA GAC GTA CAC CCG ACT CAC TAC GGT CGC GTA TGT CCA ATC GAA ACC CCT GAA GGT CCG
541-567   Glu-Arg-Ala-Gly-Phe-Glu-Val-Arg-Asp-Val-His-Pro-Thr-His-Tyr-Gly-Arg-Val-Cys-Pro-Ile-Glu-Thr-Pro-Glu-Gly-Pro-

1783-1863 AAC ATC GGT CTG ATC AAC TCT CTG TCC GTG TAC GCA CAG ACT AAC GAA TAC GGC TTC CTT GAG ACT CCG TAT CGT AAA GTG
568-594   Asn-Ile-Gly-Leu-Ile-Asn-Ser-Leu-Ser-Val-Tyr-Ala-Gln-Thr-Asn-Glu-Tyr-Gly-Phe-Leu-Glu-Thr-Pro-Tyr-Arg-Lys-Val-

1864-1944 ACC GAC GGT GTT GTA ACT GAC GAA ATT CAC TAC CTG TCT GCT ATC GAA GAA GGC AAC TAC GTT ATC GCC CAG GCG AAC TCC
595-621   Thr-Asp-Gly-Val-Val-Thr-Asp-Glu-Ile-His-Tyr-Leu-Ser-Ala-Ile-Glu-Glu-Gly-Asn-Tyr-Val-Ile-Ala-Gln-Ala-Asn-Ser-

1945-2025 AAC TTG GAT GAA GAA GGC CAC TTC GTA GAA GAC CTG GTA ACT TGC CGT AGC AAA GGC GAA TCC AGC TTG TTC AGC CGC GAC
622-648   Asn-Leu-Asp-Glu-Glu-Gly-His-Phe-Val-Glu-Asp-Leu-Val-Thr-Cys-Arg-Ser-Lys-Gly-Glu-Ser-Ser-Leu-Phe-Ser-Arg-Asp-

2026-2106 CAG GTT GAC TAC ATG GAC GTA TCC ACC CAG CAG GTG GTA TCC GTC GGT GCG TCC CTG ATC CCG TTC CTG GAA CAC GAT GAC
649-675   Gln-Val-Asp-Tyr-Met-Asp-Val-Ser-Thr-Gln-Gln-Val-Val-Ser-Val-Gly-Ala-Ser-Leu-Ile-Pro-Phe-Leu-Glu-His-Asp-Asp-

2107-2187 GCC AAC CGT GCA TTG ATG GGT GCG AAC ATG CAA CGT CAG GCC GTT CCG ACT CTG CGC GCT GAT AAG CCG CTG GTT GGT ACT
676-702   Ala-Asn-Arg-Ala-Leu-Met-Gly-Ala-Asn-Met-Gln-Arg-Gln-Ala-Val-Pro-Thr-Leu-Arg-Ala-Asp-Lys-Pro-Leu-Val-Gly-Thr-

2188-2268 GGT ATG GAA CGT GCT GTT GCC GTT GAC TCC GGT GTA ACT GCG GTA GCT AAA CGT GGT GGT GTC GTT CAG TAC GTG GAT GCT
703-729   Gly-Met-Glu-Arg-Ala-Val-Ala-Val-Asp-Ser-Gly-Val-Thr-Ala-Val-Ala-Lys-Arg-Gly-Gly-Val-Val-Gln-Tyr-Val-Asp-Ala-

2269-2349 TCC CGT ATC GTT ATC AAA GTT AAC GAA GAC GAG ATG TAT CCG GGT GAA GCA GGT ATC GAC ATC TAC AAC CTG ACC AAA TAC
730-756   Ser-Arg-Ile-Val-Ile-Lys-Val-Asn-Glu-Asp-Glu-Met-Tyr-Pro-Gly-Glu-Ala-Gly-Ile-Asp-Ile-Tyr-Asn-Leu-Thr-Lys-Tyr-

2350-2430 ACC CGT TCT AAC CAG AAC ACC TGT ATC AAC CAG ATG CCG TGT GTG TCT CTG GGT GAA CCG GTT GAA CGT GGC GAC GTG CTG
757-783   Thr-Arg-Ser-As -Gln-Asn-Thr-Cys-Ile-Asn-Gln-Met-Pro-Cys-Val-Ser-Leu-Gly-Glu-Pro-Val-Glu-Arg-Gly-Asp-Val-Leu-
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(Fig. Contd.)

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2431-2511
784-810     Ala-Asp-Gly-Pro-Ser-Thr-Asp-Leu-Gly-Glu-Leu-Ala-Leu-Gly-Gln-Asn-Met-Arg-Val-Ala-Phe-Met-Pro-Trp-Asn-Gly-...

2512-2592
811-837     AAC TTC GAA GAC TCC ATC CTC GTA TCC GAG CGT GTT GTT CAG GAA GAC CGT TTC ACC ACC ATC CAC ATT CAG GAA CTG GCG
            Asn-Phe-Glu-Asp-Ser-Ile-Leu-Val-Ser-Glu-Arg-Val-Val-Gln-Glu-Asp-Arg-Phe-Thr-Thr-Ile-His-Ile-Gln-Glu-Leu-Ala-

2593-2673
838-864     TGT GTG TCC CGT GAC ACC AAG CTG GGT CCG GAA GAG ATC ACC GCT GAC ATC CCG AAC GTG GGT GAA GCT GCG CTC TCC AAA
            Cys-Val-Ser-Arg-Asp-Thr-Lys-Leu-Gly-Pro-Glu-Glu-Ile-Thr-Ala-Asp-Ile-Pro-Asn-Val-Gly-Glu-Ala-Ala-Leu-Ser-Lys-

2674-2754
865-891     CTG GAT GAA TCC GGT ATC GTT TAC ATT GGT GCG GAA GTG ACC GGT GGC GAC ATT CTG GTT GGT AAG GTA ACG CCG AAA GGT
            Leu-Asp-Glu-Ser-Gly-Ile-Val-Tyr-Ile-Gly-Ala-Glu-Val-Thr-Gly-Gly-Asp-Ile-Leu-Val-Gly-Lys-Val-Thr-Pro-Lys-Gly-

2755-2835
892-918     GAA ACT CAG CTG ACC CCA GAA GAA AAA CTG CTG CGT GCG ATC TTC GGT GAG AAA GCC TCT GAC GTT AAA GAC TCT TCT CTG
            Glu-Thr-Gln-Leu-Thr-Pro-Glu-Glu-Lys-Leu-Leu-Arg-Ala-Ile-Phe-Gly-Glu-Lys-Ala-Ser-Asp-Val-Lys-Asp-Ser-Ser-Leu-

2836-2916
919-945     CGC GTA CCA AAC GGT GTA TCC GGT ACG GTT ATC GAC GTT CAG GTC TTT ACT CGC GAT GGC GTA GAA AAA GAC AAA CGT GCG
            Arg-Val-Pro-Asn-Gly-Val-Ser-Gly-Thr-Val-Ile-Asp-Val-Gln-Val-Phe-Thr-Arg-Asp-Gly-Val-Glu-Lys-Asp-Lys-Arg-Ala

2917-2997
946-972     CTG GAA ATC GAA GAA ATG CAG CTC AAA CAG GCG AAG AAA GAC CTG TCT GAA GAA CTG CAG ATC CTC GAA GCG GGT CTG TTC
            Leu-Glu-Ile-Glu-Glu-Met-Gln-Leu-Lys-Gln-Ala-Lys-Lys-Asp-Leu-Ser-Glu-Glu-Leu-Gln-Ile-Leu-Glu-Ala-Gly-Leu-Phe

2998-3078
973-999     AGC CGT ATC CGT GCT GTG CTG GTA GCC GGT GGC GTT GAA GCT GAG AAG CTC GAC AAA CTG CCG CGC GAT CGC TGG CTG GAG
            Ser-Arg-Ile-Arg-Ala-Val-Leu-Val-Ala-Gly-Gly-Val-Glu-Ala-Glu-Lys-Leu-Asp-Lys-Leu-Pro-Arg-Asp-Arg-Trp-Leu-Glu

3079-3159
1000-1026   CTG GGC CTG ACA GAC GAA GAG AAA CAA AAT CAG CTG GAA CAG CTG GCT GAG CAG TAT GAC GAA CTG AAA CAC GAG TTC GAG
            Leu-Gly-Leu-Thr-Asp-Glu-Glu-Lys-Gln-Asn-Gln-Leu-Glu-Gln-Leu-Ala-Glu-Gln-Tyr-Asp-Glu-Leu-Lys-His-Glu-Phe-Glu

3160-3240
1027-1053   AAG AAA CTC GAA GCG AAA CGC CGC AAA ATC ACC C*AG GGC GAC GAT CTG GCA CCG GGC GTG CTG AAG ATT GTT AAG GTA TAT
            Lys-Lys-Leu-Glu-Ala-Lys-Arg-Arg-Lys-Ile-Thr-Gln-Gly-Asp-Asp-Leu-Ala-Pro-Gly-Val-Leu-Lys-Ile-Val-Lys-Val-Tyr

3241-3321
1054-1080   CTG GCG GTT AAA CGC CGT ATC CAG C*CT GGT GAC AAG ATG GCA GGT CGT CAC GGT AAC AAG GGT GTA ATT TCT AAG ATC AAC
            Leu-Ala-Val-Lys-Arg-Arg-Ile-Gln-Pro-Gly-Asp-Lys-Met-Ala-Gly-Arg-His-Gly-Asn-Lys-Gly-Val-Ile-Ser-Lys-Ile-Asn-

3322-3402
1081-1107   CCG ATC GAA GAT ATG CCT TAC GAT GAA AAC GGT ACG CCG GTA GAC ATC GTA CTG AAC CCG CTG GGC GTA CCG TCT CGT ATG
            Pro-Ile-Glu-Asp-Met-Pro-Tyr-Asp-Glu-Asn-Gly-Thr-Pro-Val-Asp-Ile-Val-Leu-Asn-Pro-Leu-Gly-Val-Pro-Ser-Arg-Met-

3403-3483
1108-1134   AAC ATC GGT CAG ATC CTC GAA ACC CAC C*TG GGT ATG GCT GCG AAA GGT ATC GGC GAC AAG ATC AAC GCC ATG CTG AAA CAG
            Asn-Ile-Gly-Gln-Ile-Leu-Glu-Thr-His-Leu-Gly-Met-Ala-Ala-Lys-Gly-Ile-Gly-Asp-Lys-Ile-Asn-Ala-Met-Leu-Lys-Gln-

3484-3564
1135-1161   CAG CAA GAA GTC GCG AAA CTG CGC GAA TTC ATC CAG CGT GCG TAC GAT CTG GGC GCT GAC GTT CGT CAG AAA GTT GAC CTG
            Gln-Gln-Glu-Val-Ala-Lys-Leu-Arg-Glu-Phe-Ile-Gln-Arg-Ala-Tyr-Asp-Leu-Gly-Ala-Asp-Val-Arg-Gln-Lys-Val-Asp-Leu-

3565-3645
1162-1188   AGT ACC TTC AGC GAT GAA GAA GTT ATG CGT CTG GCT GAA AAC CTG CGC AAA GGT ATG CCA ATC GCA ACG CCG GTG TTC GAC
            Ser-Thr-Phe-Ser-Asp-Glu-Glu-Val-Met-Arg-Leu-Ala-Glu-Asn-Leu-Arg-Lys-Gly-Met-Pro-Ile-Ala-Thr-Pro-Val-Phe-Asp-

3646-3726
1189-1215   GGT GCG AAA GAA GCA GAA ATT AAA GAG CTG CTG AAA CTT GGC GAC CTG CCG ACT TCC GGT CAG ATC CGC CTG TAC GAT GGT
            Gly-Ala-Lys-Glu-Ala-Glu-Ile-Lys-Glu-Leu-Leu-Lys-Leu-Gly-Asp-Leu-Pro-Thr-Ser-Gly-Gln-Ile-Arg-Leu-Tyr-Asp-Gly-

3727-3807
1216-1242   CGC ACT GGT GAA CAG TTC GAG CGT CCG GTA ACC GTT GGT TAC ATG TAC ATG CTG AAA CTG AAC CAC C*TG GTC GAC GAC AAG
            Arg-Thr-Gly-Glu-Gln-Phe-Glu-Arg-Pro-Val-Thr-Val-Gly-Tyr-Met-Tyr-Met-Leu-Lys-Leu-Asn-His-Leu-Val-Asp-Asp-Lys-

3808-3888
1243-1269   ATG CAC GCG CGT TCC ACC GGT TCT TAC AGC C*TG GTT ACT CAG CAG CCG CTG GGT GGT AAG GCA CAG TTC GGT GGT CAG CGT
            Met-His-Ala-Arg-Ser-Thr-Gly-Ser-Tyr-Ser-Leu-Val-Thr-Gln-Gln-Pro-Leu-Gly-Gly-Lys-Ala-Gln-Phe-Gly-Gly-Gln-Arg-

3889-3969
1270-1296   TTC GGG GAG ATG GAA GTG TGG GCG CTG GAA GCA TAC GGC GCA GCA TAC ACC CTG CAG GAA ATG CTC ACC GTT AAG TCT GAT
            Phe-Gly-Glu-Met-Glu-Val-Trp-Ala-Leu-Glu-Ala-Tyr-Gly-Ala-Ala-Tyr-Thr-Leu-Gln-Glu-Met-Leu-Thr-Val-Lys-Ser-Asp-

3970-4050
1297-1323   GAC GTG AAC GGT CGT ACC AAG ATG TAT AAA AAC ATC GTG GAC GGC AAC CAT CAG ATG GAG CCG GGC ATG CCA GAA TCC TTC
            Asp-Val-Asn-Gly-Arg-Thr-Lys-Met-Tyr-Lys-Asn-Ile-Val-Asp-Gly-Asn-His-Gln-Met-Glu-Pro-Gly-Met-Pro-Glu-Ser-Phe-

4051-4129
1324-1342   AAC GTA TTG TTG AAA GAG ATT CGT TCG CTG GGT ATC AAC ATC GAA CTG GAA GAC GAG TAA TTC TCG CTC AAA CAG GTC A
            Asn-Val-Leu-Leu-Lys-Glu-Ile-Arg-Ser-Leu-Gly-Ile-Asn-Ile-Glu-Leu-Glu-Asp-Glu TER

4130-4210
1-8         CTG CTG TCG GGT TAA AAC CCG GCA GCG GAT TGT GCT AAC TCC GAC GGG AGC AAA TCC GTG AAA GAT TTA TTA AAG TTT CTG
                                                                                        Met-Lys-Asp-Leu-Leu-Lys-Phe-Leu-

4211-4291
9-35        AAA GCG CAG ACT AAA ACC GAA GAG TTT GAT GCG ATC AAA ATT GCT CTG GCT TCG CCA GAC ATG ATC CGT TCA TGG TCT TTC
            Lys-Ala-Gln-Thr-Lys-Thr-Glu-Glu-Phe-Asp-Ala-Ile-Lys-Ile-Ala-Leu-Ala-Ser-Pro-Asp-Met-Ile-Arg-Ser-Trp-Ser-Phe-

4292-4372
36-62       GGT GAA GTT AAA AAG CCG GAA ACC ATC AAC TAC CGT ACG TTC AAA CCA GAA CGT GAC GGC CTT TTC TGC GCC CGT ATC TTT
            Gly-Glu-Val-Lys-Lys-Pro-Glu-Thr-Ile-Asn-Tyr-Arg-Thr-Phe-Lys-Pro-Glu-Arg-Asp-Gly-Leu-Phe-Cys-Ala-Arg-Ile-Phe-

4373-4453
63-89       GGG CCG GTA AAA GAT TAC GAG TGC CTG TGC GGT AAG TAC AAG CGC CTG AAA CAC CGT GGC GTC ATC TGT GAG AAG TGC GGC
            Gly-Pro-Val-Lys-Asp-Tyr-Glu-Cys-Leu-Cys-Gly-Lys-Tyr-Lys-Arg-Leu-Lys-His-Arg-Gly-Val-Ile-Cys-Glu-Lys-Cys-Gly-

4454-4534
90-116      GTT GAA GTG ACC CAG ACT AAA GTA CGC CGT GAG CGT ATG GGC CAC ATC GAA CTG GCT TCC CCG ACT GCG CAC ATC TGG TTC
            Val-Glu-Val-Thr-Gln-Thr-Lys-Val-Arg-Arg-Glu-Arg-Met-Gly-His-Ile-Glu-Leu-Ala-Ser-Pro-Thr-Ala-His-Ile-Trp-Phe-

4535-4615
117-143     CTG AAA TCG CTG CCG TCC CGT ATC GGT CTG CTG CTC GAT ATG CCG CTG CGC GAT ATC GAA CGC GTA CTG TAC TTT GAA TCC
            Leu-Lys-Ser-Leu-Pro-Ser-Arg-Ile-Gly-Leu-Leu-Leu-Asp-Met-Pro-Leu-Arg-Asp-Ile-Glu-Arg-Val-Leu-Tyr-Phe-Glu-Ser-

4616-4696
144-170     TAT GTG GTT ATC GAA GGC GGT ATG ACC AAC C*TG GAA CGT CAG CAG ATC CTG ACT GAA GAG CAG TAT CTG GAC GCG CTG GAA
            Tyr-Val-Val-Ile-Glu-Gly-Gly-Met-Thr-Asn-Leu-Glu-Arg-Gln-Gln-Ile-Leu-Thr-Glu-Glu-Gln-Tyr-Leu-Asp-Ala-Leu-Glu-

4697-4714
171-176     GAG TTC GGT GAC GAA TTC
            Glu-Phe-Gly-Asp-Glu-Phe
```

FIG. 2. The nucleotide sequence of the rpoBC segment, the total amino acid sequence of the β-subunit and the N-terminal amino acid sequence of the β′-subunit of *E. coli* RNA polymerase. The restriction EcoRI cleavage sites dividing the fragments EcoRI-G, EcoRI-C and EcoRI-F are situated between nucleotides 640–641 and 3508–3509. Here and in Fig. 3 the nucleotide sequence of the complementary DNA chain, equal to the sequence of mRNA, is given. The underlined amino acid sequences are those the structure of which has been determined from analysis of corresponding peptides. C* refer to 5-methylcytidine residues.

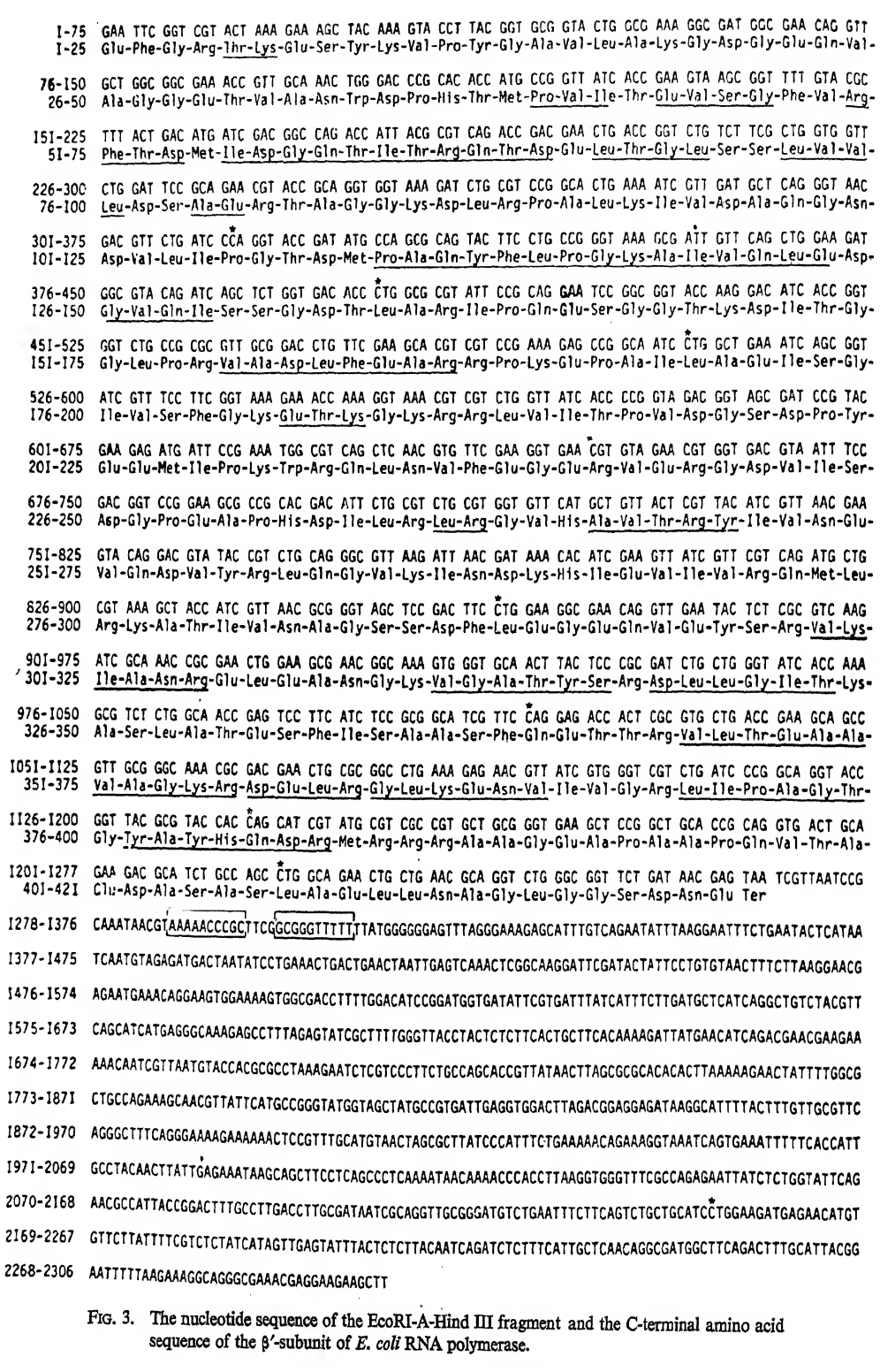

FIG. 3. The nucleotide sequence of the EcoRI-A-Hind III fragment and the C-terminal amino acid sequence of the β′-subunit of *E. coli* RNA polymerase.

γ-azidoanilidate of GTP as an initiation substrate and various incomplete sets of nucleoside triphosphates, one of which was radioactively labelled (Fig. 4).

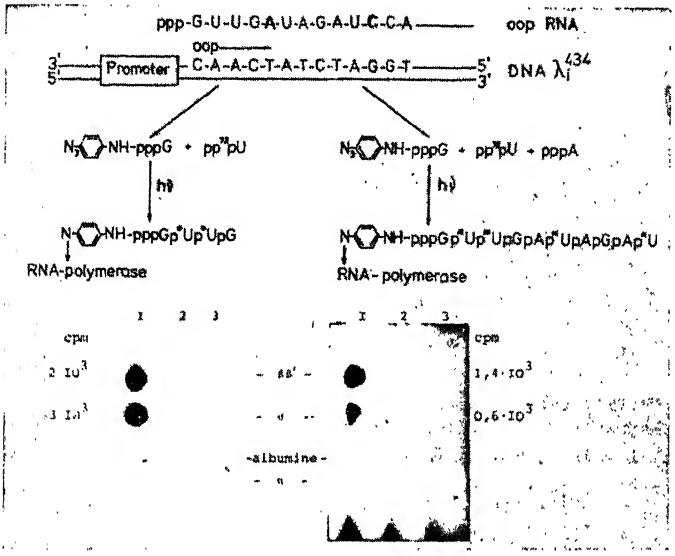

FIG. 4. Crosslinking of the RNA polymerase with photoreactive RNA in transcribing complex: (1) after irradiation; (2) without irradiation; and (3) the complex was destroyed before irradiation.

Maximum tetranucleotide can be synthesized in the presence of γ-azidoanilidate of GTP and UTP, while decanucleotide in the presence of γ-azidoanilidate of UTP and ATP. Obviously radioactive label covalently bound to RNA polymerase upon illumination at $\lambda \geqslant 290$nm may be due only to the oligonucleotides synthesized in the system, rather than to initial substrates, because the γ-azidoanilidate is not radioactive, whereas the pp^{32} pU is not photoreactive. After photoaffinity labelling the β, β′ and σ-subunits become radioactive in the presence of two triphosphates, but in the case of three substrates radioactivity predominates in β, β′-subunits (Fig. 4). These data presumably reflect the shift of the 5′-end of the nascent oligonucleotide within the transcribing complex when its length grows continuously up to decanucleotide (Sverdlov *et al.*, 1979).

A similar methodology was employed to establish the subunits contacting with the 3′-OH end of the growing RNA. Affinity modification was made by the oligonucleotides being synthesized *in situ*. They contained photoreactive 5-halogenopyrimidine residues in the vicinity of 3′-OH end. In the presence of GPU (the primer) 5-IU or 5-BrU and | α—^{32}P | GTP (substrates) and the above-mentioned λ_{imm} DNA fragment (template) only σ-subunit incorporated the radiactive label upon UV-irradiation (Sverdlov *et al.*, 1980). Hence, the role of the σ-subunit is not limited by recognition of the promoter, it may also directly participate in RNA synthesis initiation.

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Organic Chemistry

PROGRESS IN THE CHEMISTRY OF SYNTHETIC MACROCYCLIC COMPLEXONES AND THEIR ANALOGUES

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The problems of progress in chemistry of synthetic macrocyclic complexones and their analogues are considered. Considerable improvement of the methods of syntheses of crown ethers, cryptands and other macrocycles, obtaining new structural types, considerable expansion of ways of using macrocyclic complexones in science and technology are illustrated by a number of examples. Author gives attention to the data obtained at the Physico-Chemical Institute of the Academy of Sciences of Ukrainian SSR as a result of investigations realized by himself and his colleagues.

Keywords: **Macrocyclic Complexones; Crown Ethers; Cryptands; Chiral Crown Ethers and Cryptands; Dividing of Racemates; Ligands Specific for Ions**

INTRODUCTION

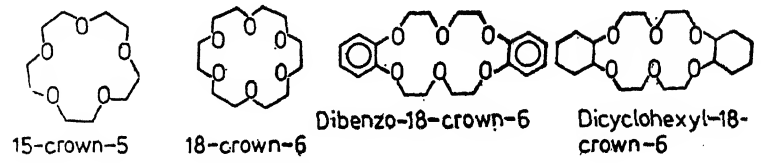
AT the end of 70s, the chemistry of synthetic macrocyclic complexones and their analogues had achieved a new success. It is evident now that this new branch of chemistry has great significance not from the theoretical point of view alone. Practical possibilities of chemistry of synthetic macrocyclic complexones have appeared very important and ways of their applications in practice are numerous and varied.

It is clear that the chemistry of synthetic macrocyclic complexones and their analogues is a new direction in modern chemistry which suggests an attractive outlook for solving many problems on the new level with low energy expense, convenient and simple realization of processes.

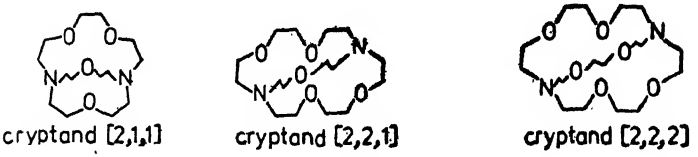
Usually, among synthetic macrocylic complexones we distinguish crown ethers and cryptands, synthesis of which had begun in 1967–68 (Pedersen, 1967 *a*, *b*, 1970).

These compounds and numerous other similar objects form highly lipophillic complexes with series of metals and also with organic molecules like natural membrane-active complexones, ionophores (valinomycin, ennatin, nonactine and others). By analogy with the last, some crown ethers (but not cryptands) possess ability to transport ions of alkali and alkali-earth metals across biological membranes. The discovery of the unique complexing and solvatation properties of synthetic cyclic polyethers, their analogues and complexes on their base opened a prospect to apply them in various branches of science and technology in the form of highly efficient

catalysts, extragents, biologically active substances, drugs, analytical reagents and new materials.



Crown ethers



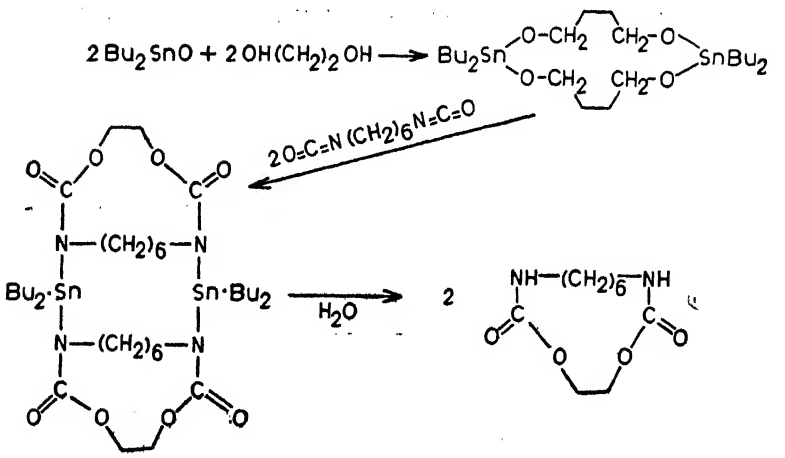
Cryptands

In studying natural membrane-active complexones (ionophores), Soviet Scientific School of Bioorganic Chemists with Academician Yu. A. Ovchinnikov at the head keeps the leading place in the world. Their investigations have opened a new page in the history of physico-chemical biology. The works of Soviet scientists led to the discovery of nature of two ion-transport mechanisms across membranes by means of ionophores and across channels or ion conductors (Ovchinnikov *et al.*, 1974; and Ovchinnikov, 1978). Highly useful results have been seen in studying the channels, which have not been investigated enough until now.

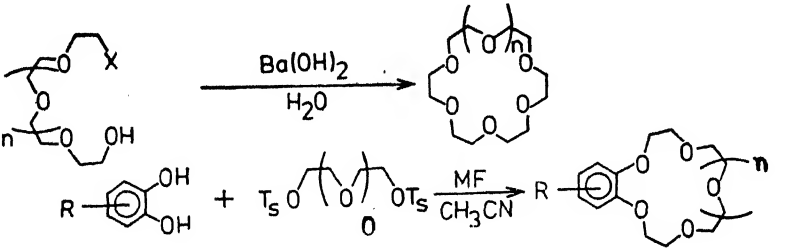
During the last few years, the chemistry of synthetic macrocyclic complexones and their analogues, which should be considered as an independent scientific branch, has been constantly growing both in creating new and synthetic macrocyclic models and their open chain analogues and in their application to various branches of science and technology. Beginning at first in the direction of scientific investigation on creation of the simplified models of the natural ionophores, this branch of synthetic chemistry acquired quite an independent significance. Since 1977, investigations in the field of the chemistry of synthetic macrocyclic complexones have developed at the Physico-Chemical Institute of the Academy of Sciences of the Ukrainian SSR (Odessa, USSR), In this paper, we discuss both the results obtained according to our investigation and study in 1977–80 and also the data received by other authors from different countries. All the results indicate great progress in this new branch of chemistry.

Investigations

Recently, the new convenient methods of synthesis of crown ethers have been worked out by means of intermediates of tin organic compounds (Shanzer *et al.*, 1980). This method is interesting because it allows to depart from traditional (in synthesis of macrocycles) method of high dilution. From the following scheme it is seen that the above mentioned method is realized comparatively simply:



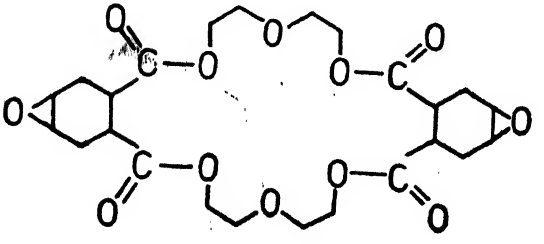
Similar methods, based on the preliminary inclusion of metal into macrocycle with its following removal and base, in a greater degree on the effect of template catalysis, are widely used both in synthesis of crown ethers and another macrocycles (Kulstad & Malmsten, 1980; Mandolini & Masci, 1979; and Reinhoudt *et al.*, 1979).



The common practice in developing synthesis of macrocycles in the eighties is to refuse primary methods used at the end of the sixties and the beginning of seventies and to apply catalytic methods which do not demand high dilution. This practice has, besides being purely chemical, is also economical. Synthetic macrocyclic complexones must be cheaper now and more accessible.

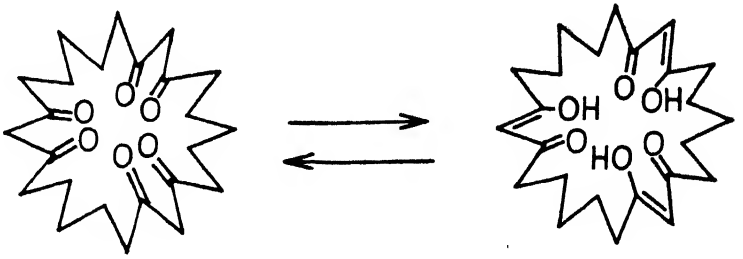
The second achievement has been a search for new structures of synthetic macrocycles with significant possibilities of complexing.

Recently, a macrocyclic ether, containing the oxyrane groups has been synthesized in our laboratory (Bogatsky *et al.*, 1980 *a*).



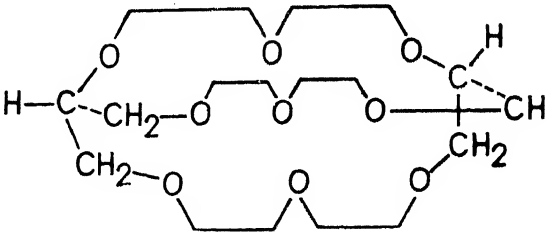
It is not difficult to understand that the oxyrane groups can be used for introduction of any functional groups into the similar macrocycle.

The macrocyclic hexaketone with beta-position of ketonic group was synthesized in 1979 in Japan (Tabushi *et al.*, 1979). This compound conceals many possibilities. An interesting example is shown below:



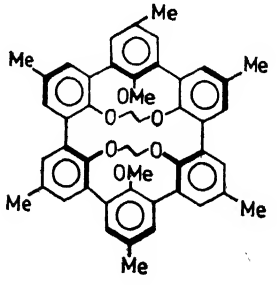
This hexaketon almost quantitatively extracts ion UO from the diluted aqueous solution into organic medium. It is 260 times effective than dicyclohexo-18-crown-6.

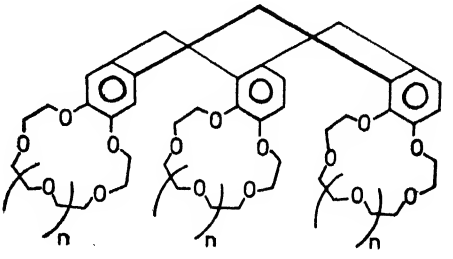
Another important achievement is the synthesis of stereoisomeric non-nitrogen cryptands based on glycerine (Haines & Karntiang, 1979). This non-nitrogen or cryptand may appear (like crown ether) as an analogue of ionophores and its role in complexing is expected to be very important.



Spherand synthesized by D. Cram is a polyhedral compound: it led to possibilities of application in complexing with cations (Cram *et al.*, 1979). A seven-staged synthesis of spherand was carried out with a total yield of about 1.2 per cent.

It is important here to note a series of works by F. Vögtle on the obtaining of the so-called "multistaged crown ether missiles" (Frensch & Vögtle, 1979). They present mini-fragments immobilized crown ethers and in future they will be used for the so-called assembly of structures containing macrocyclic fragments. We present here the formula of synthesized crown-ether with triveratiilene skeleton:



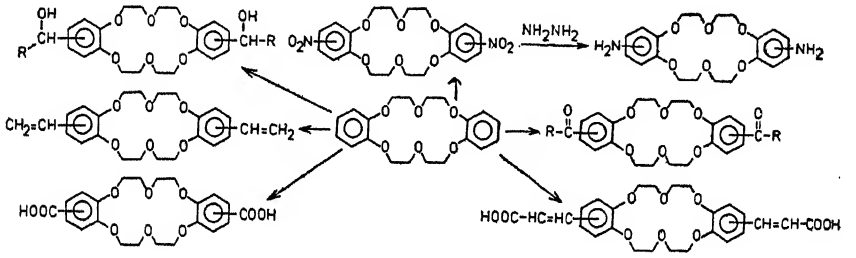


A large number of examples of new synthetic finds can be shown but for lack of space and time, I would like to show only that the present chemists equipped by modern methods and instruments, modern theories and experiments and methods of modelling, will achieve summits in molecular design creating the most fantastic and complex structures necessary for definite purposes.

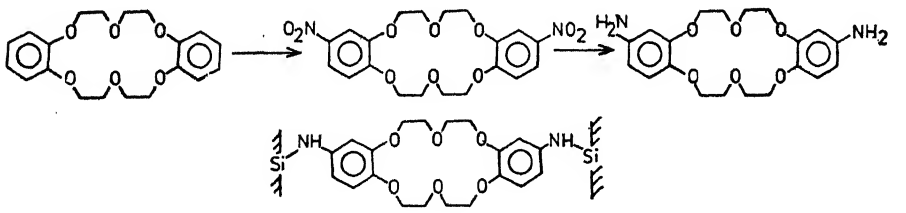
In this connection, works on implantation of synthetic ionophores to inorganic and organic matrices, "immobilization" of synthetic macrocycles are very interesting.

For the first time immobilization of derivatives of dibenzo-18-crown-6 on inorganic matrices was carried out at our Institute (Bogatsky *et al.*, 1979). Research on modification of dibenzo-18-crown-6 and obtaining a large number of its derivatives was undertaken by us.

Many of these derivatives are highly useful for new syntheses and for obtaining new materials, based on the following derivatives:



On the basis of diamino dibenzo-18-crown-6, we have carried out "immobilization" of this object on silochrome by the method, the essence of which is opened below:

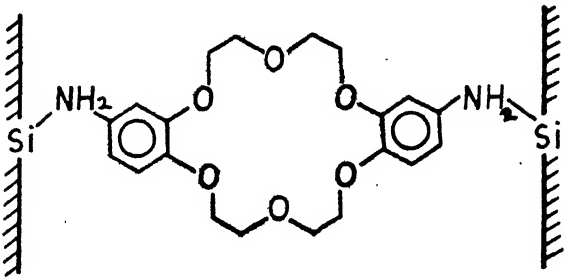


As a result, the highly efficient catalyst of nucleophilic reactions was obtained. That is seen from Table I where the results of the model reaction are presented.

This catalyst can be used many times. Immobilization of macrocyclic complexones on the organic matrices including films repeatedly was carried out by many researchers who gave dissimilar results (Blasins *et al.*, 1977; and Regen, 1979). Such immobilization was realized by means of preliminary obtaining of divinyl-derivative of dibenzo-18-crown-6 which was monomer and polymerization (or co-polymerization) on the basis of this monomer. These polymeric crown ethers obtained at our

TABLE I

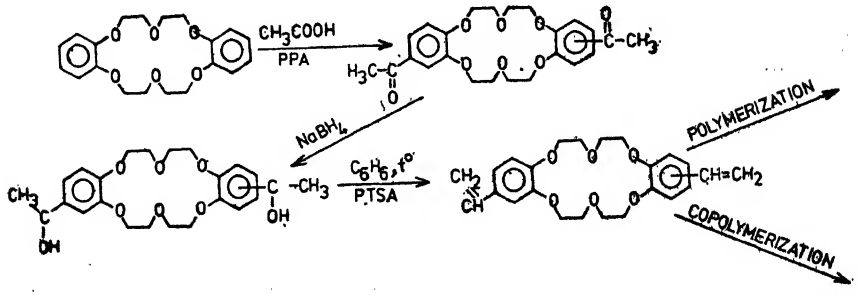
Application of immobilized dibenzo-18-crown-6 as a catalyst of nucleophilic reactions



$$CH_3COOK + RBr \xrightarrow{Kt} CH_3COOR + KBr$$

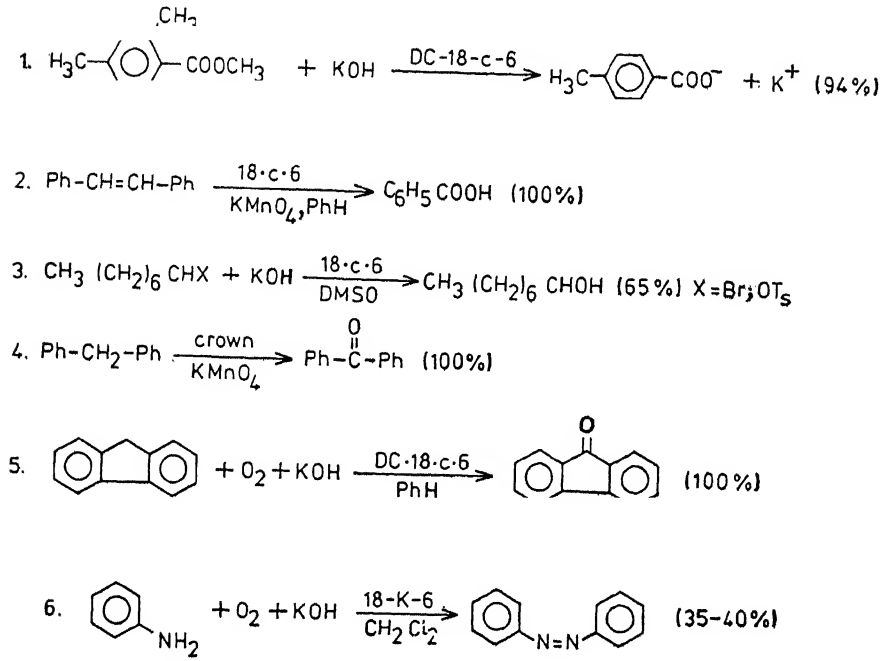
Substratum[mole/1	CH_3COOH mole/1	Catalyst mole $.10^{-4}$	T °C	Reaction time, hrs	Yield, %
0.22	0.40	1.0	83	2	100
0.22	0.40	0.0	83	4.5	100
2.20	3.33	0.46	20	88	95
0.22	0.33	1.0	83	9	80

Institute have been more interesting than substances obtained by other authors before comparative crown ether fragments were involved into polymeric material mechanically.



Every year there is an increase in the application of synthetic macrocyclic complexones and their analogues in organic synthesis in the form of catalysts, carriers and dividers of ionic pairs. This has led to new directions and new methods of synthesis, chemical processes, new control method of stereoselective reactions, etc.

Some examples quoted according to the reference (Gokel & Dürst, 1976) are shown on the following scheme:



The effect of the first reaction was the splendid saponification of Viktor Meyer ester, popular example of steric hindrance. The effect was excited by attack of non-solvated anion, that is the lesser reagent.

In the second and third reactions as above liposoluble complex of cation of potassium permanganate in benzene was used to ensure an effective and soft oxidization by non-solvated anion with high yields. Similar effects were observed in the 4-th 5-th and 6-th reactions. The following scheme demonstrates continuation of the above series of reactions:

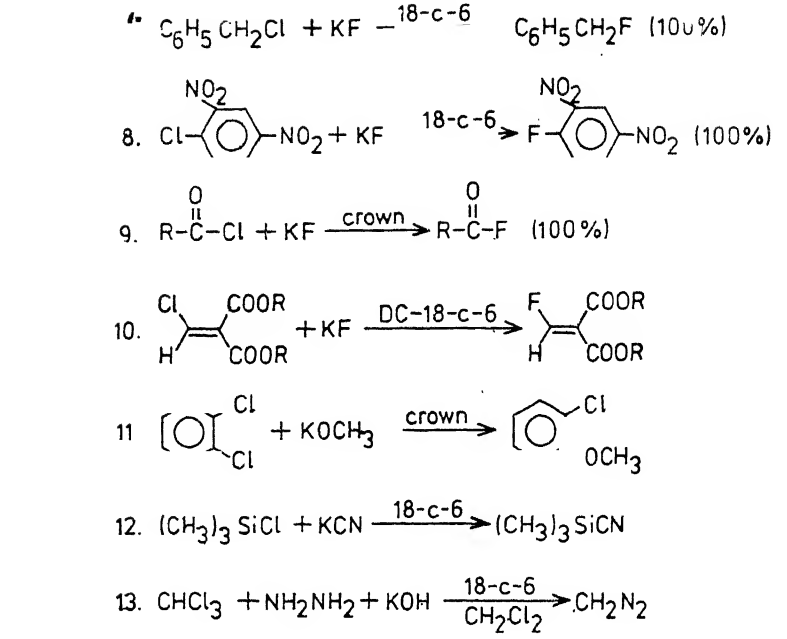
Soft fluorization was possible only when using crown ethers which "seized" cation of potassium. The reactions 7–10 have an attack of non-solvated anion of fluor under soft conditions. Similar effects were observed under reactions 11–13.

The above enumerated chemical processess can become tomorrow the base of new technological processes more convenient and economical than the present processes.

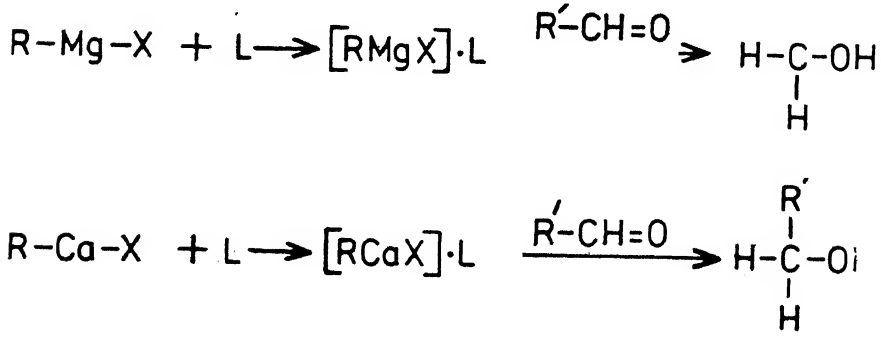
I have already mentioned about some new types of crown ethers synthesized at our Institute, about methods of immobilization of crown ethers used by our chemists and about other works. All the works I have mentioned have been carried out in close cooperation with Odessa State University and many other Research Institutes and Universities (Bogatsky *et al.*, 1979, 1980 *a, b, c, d, e, f;* and Lukyaneko *et al.*, 1980, 1981).

Developing new ideas on the application of macrocyclic complexones in organic synthesis, we have solved two difficult problems in 1979 and 1980:

The first one was carrying out Grignard reaction in the presence of macrocyclic complexones.



We find that magnesium and calcium alkyl halides form reactivity complexes with Grignard reagents. The letter "L" is for crown ether (Bogatsky *et al.*, 1980 *f*).



Reactions above are smooth and effective. In addition, they do not require di-ethyl ether or tetrahydrofuran. The catalytic quantity of crown ether is enough to carry out reactions in comparatively high boiling solvents, such as toluene, xylene, etc. (Table II).

In other words thanks to this modification with Grignard reagents the most essential demerit of classical methods, namely, necessity to work with inflammable and easily explosive substances has been removed.

The second problem on the synthetic macrocyclic complexones investigated at our Institute is to apply crown ethers for controlling stereochemistry of reactions.

TABLE II

Conditions in the Grignard reaction in presence of catalytic quantities of crown-ethers

$$RX + C{=}O \xrightarrow[\text{crown ethers (3–5 \%)}]{M} C(R)OH$$

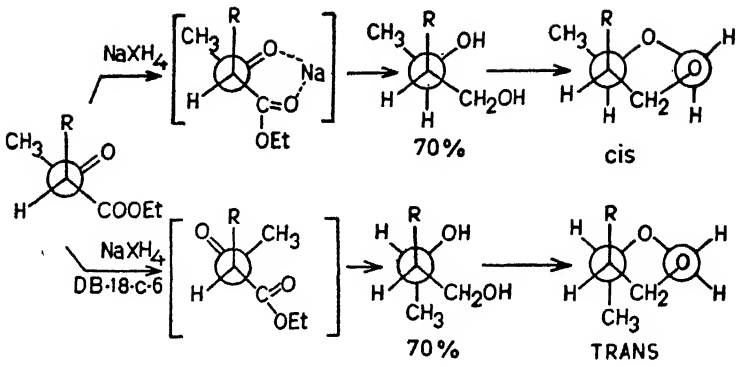
RX	C=O	M	Solvent	M. RX / C—O	Yield of alcohol % (*a*)	Yield of alcohol in the absence of crown ether (*b*)
CH_3I	$(CH_3)_2CHCHO$	Mg	Diethyl ether	1.5 : 1	80 (97)	66
CH_3I	$(CH_3)_2CHCHO$	Mg	Benzene	1 : 1	45 (78)	—
CH_3I	$(CH_3)_2CHCHO$	Mg	Hexane	2 : 1	80 (90)	—
CH_3I	C_6H_5CHO	Ca	Diethyl ether	1 : 1	71 (83)	55
CH_3I	C_6H_5CHO	Mg	Benzene	1 : 1	47 (83)	—
CH_3I	C_6H_5CHO	Mg	Benzene	2 : 1	88 (96)	—
CH_3I	C_6H_5CHO	Mg	Heptane	2 : 1	87 (95)	—
CH_3I[b]	$CH_3COOC_2H_5$	Ca	Benzene	2 : 1	54 (89)	—
CH_3I[b]	$CH_3COOC_2H_5$	Mg	O-xylene	4 : 1	80 (95)	—
C_2H_5Br	C_6H_5CHO	Mg	Diethyl ether	1 : 1	79 (93)	78
C_2H_5Br	C_6H_5CHO	Mg	Benzene	1 : 1	52 (92)	—
C_2H_5Br	C_6H_5CHO	Ca	Toluene	2 : 1	80 (96)	—
C_2H_5Br	$CH_3COC_6H_5$	Mg	Diethyl ether	2 : 1	81 (90)	67
C_2H_5Br	$CH_3COC_6H_5$	Mg	Benzene	1 : 1	45 (87)	—
C_2H_5Br	$CH_3COC_6H_5$	Ca	Hexane	2 : 1	78 (92)	—
C_6H_5Br	$(CH_3)_2CHCHO$	Mg	Diethyl ether	2 : 1	80 (95)	68
C_6H_5Br	$(CH_3)_2CHCHO$	Mg	Benzene	1 : 1	40 (83)	—
C_6H_5Br	$(CH_3)_2CHCHO$	Ca	Toluene	2 : 1	81 (95)	—

a) Yield of alcohol, calculated on carbonyl compounds reacted, is shown in brackets
b) Product of reaction $(CH_3)_3COH$

In the next scheme, two stereochemical ways of reduction of isopropylacetoacetate by sodium boron hydride are shown: top scheme-no crown ether. It is known that reduction processes of carbonyl compounds are stereochemically controlled by Cram's rule. Our variant of this rule describes the principal conformation where reduction is for the case when groups are capable of forming complexes (Morrison & Mosher, 1973). The conformations, as a rule, are stereochemically different:

When sodium boron hydride interacts with isopropylacetoacetate in accordance with Cram's rule, the initial conformation, in square brackets, is realized. Hence, the reagent attack follows in the direction shown by arrows: 70 per cent yield of erythroisomer of 4, 5-substituted 1, 3-dioxane is formed under conditions excepting Walden inversion (Bogatsky *et al.*, 1975). This scheme was known long ago. We added crown ether to the reaction mixture.

The crown ether is complexed with sodium forming a more stable complex. Then the conformation predicted by the variant according to Cram's rule for the case without complexes becomes the primary initial conformation.



Hence, there are other directions of attack and the yield makes up 70 per cent for threo-isomer, 1, 3-diol and from it trans-4, 5-disubstituted 1, 3-dioxane.

Therefore, one can change directions of stereospecific and stereodirectional reactions by means of crown ethers and other synthetic macrocyclic complexones.

Several other authors have published results in the analogous data (Glass *et al.*, 1980; Loupy *et al.*, 1978; and Shida & Ando, 1979). The application of crown ethers and cryptands in stereochemistry is one of the fascinating points of chemistry of the mentioned substances. Probably, dividing of racemates is the most interesting problem in this branch of chemistry. Every chemist knows all the complications of this task. We gained the impression that using chiral crown ethers and cryptands this problem can be solved quite simply.

In this case the principle of dividing racemates is similar to that based on obtaining diastereomers. Its distinction lies in the fact that the coordinated complexes are formed, but not the covalent compounds between the corresponding enantiomers are easily divided and easily regenerated.

Cram's chiral ligand is shown in Fig. 1. The object of dividing is hydrochloric

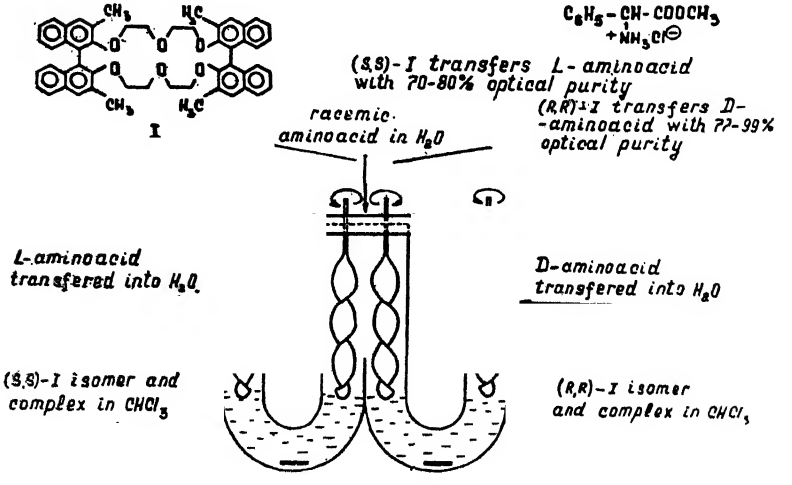


FIG. 1. Scheme of dividing and the apparatus for dividing racemic mixtures of enantioisomers of aminoacids according to Donald Cram.

salt of amino acid; and the apparatus where the dividing is carried out is shown in Fig. 1. It is important to know that the chiral carrier is not practically spent and all the time the chiral carrier is in the reaction zone. Some attempts have also been made to divide stereoisomers in columns with the chiral carrier inoculated to the inorganic material.

The synthetic macrocyclic complexones are widely applied in processes of separation mixtures and extracts of valuable substances. Some examples can be cited here:

There is a lot of information about dividing isotopes of lithium sodium potassium, calcium, etc., in the presence of cryptands (Heumann & Schiefer, 1980; Jepson & Dewitt, 1976; Jepson & Shockley, 1979; and Knöchel & Wilken, 1976). These results are important for practical use.

It is known that addition of only some percentages of 24-crown-8-ethers into the solution of tributylphosphate in kerosene increases 200 times extraction degree by this reagent and allows to extract 99.9 per cent of cesium and strontium from waste waters of atomic industry (Gerow & Davies, 1979).

It is shown that additives of dicyclohexyl-18-crown-6 and sulphonic acid increase 300 times the extraction degree of nitrates U (IV) and Eu (McDowell & Shoun, 1978).

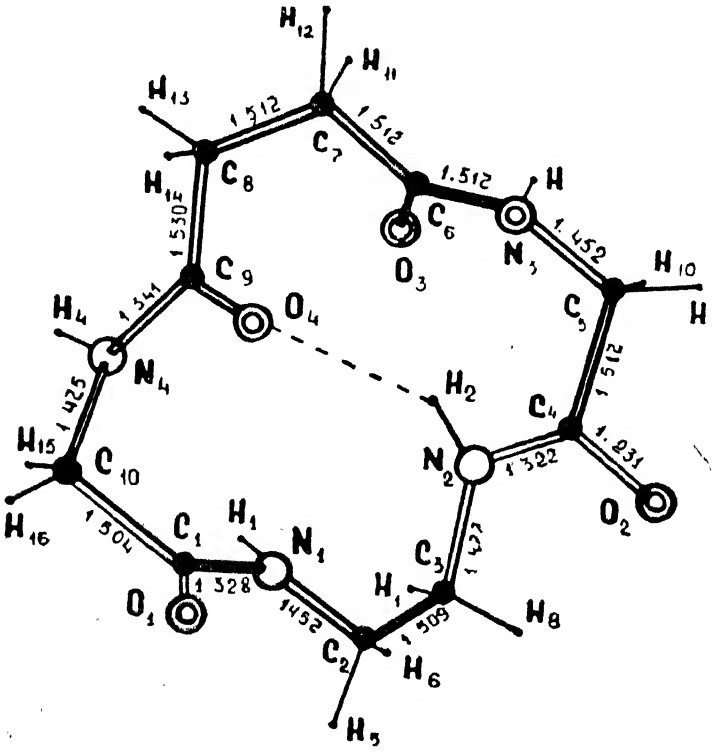


FIG. 2. Determination of the structure of macrocyclic tetraamid by method of X-ray structure analysis.

The separation of light lanthanoids (Eu, Ce, Pr, Nd, Pm, Sm) from heavy lanthonoids (Eu, Gd, Tb, Dy, Ho, Er, Tm, Yb, Lu) under complex-forming with dibenzo-18-crown-6 has been done in the U.S.A.

Cook and Montgomery (1978) have processed data about the significant increase of anionic polymerization rate of styrene and isoprene catalyzed by n-butyl lithium by means of additives of 15-crown-5 and 12-crown-4.

However, the search for specific ligands for ions of lithium, sodium, calcium, transition metals, anions, etc., remains a problem. In this connection, structural stereo-chemical investigations in the field of chemistry of macrocyclic complexones, quantum-chemical calculations, modelling various types are very important, which are carried out actively at our Institute.

More than 20 new original macrocycles have already been investigated by means of X-ray structural analysis and the exact data have been obtained about their configuration and conformation (Botoshansky *et al.*, 1980; and Malinovsky *et al.*, 1981). The example, illustrating the results of this work, is presented in Fig. 2. This figure shows the results of the X-ray analysis of the configuration and conformation of one macrocyclic tetramide, synthesized at our Institute. This work is fulfilled in close cooperation with a physicist from the Institute of Applied Physics of the Moldavian Academy of Sciences.

The Theoretical Chemistry Group of our Institute makes good progress in research calculations. For modelling, we use widely CPK and Dreiding models to compare the physico-chemical measurements.

It allowed us to achieve certain successes in the synthesis of macrocyclic ligands for calcium ion. The information about it is given in Fig. 3. It is well noted that a 36-membered macrocycle, containing ester groups, appeared specific for calcium ion.

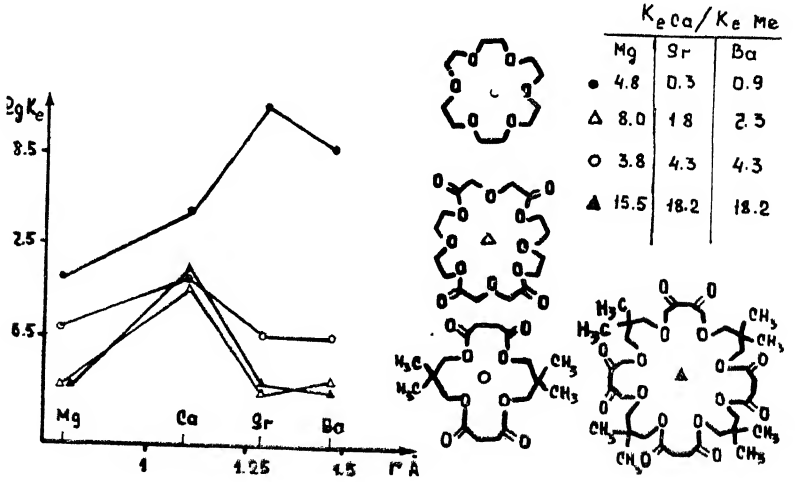
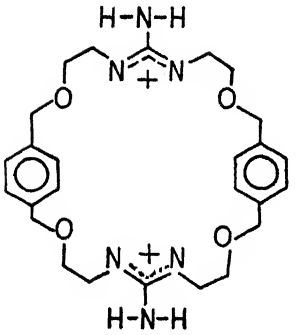
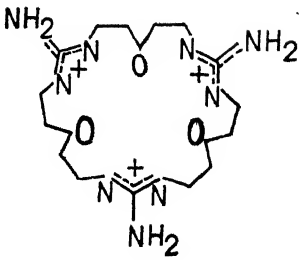


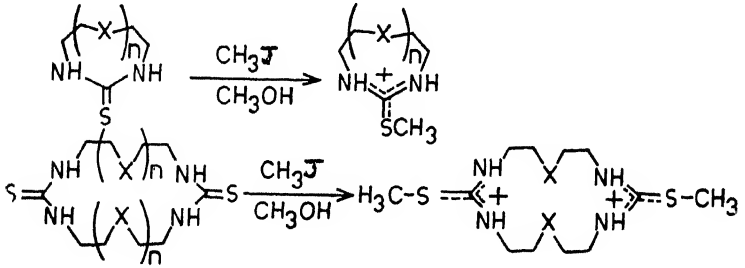
FIG. 3. Selective extraction of calcium ions by macrocyclic esters.

Recently, J. M. Lehn (France) (1977) have synthesized macrocyclic ligands for anions containing guanidine fragments. It appeared very specific for PO_4^{3-} ion (Dietrich *et al.*, 1978).





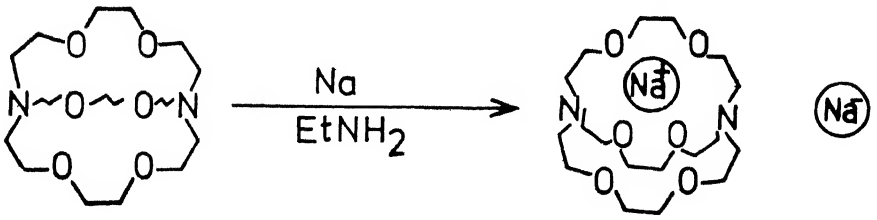
At the same time the author also has synthesized similar ligands on the basis of isothiourea. It is obvious that both types of macrocycles are isoelectronic analogues.



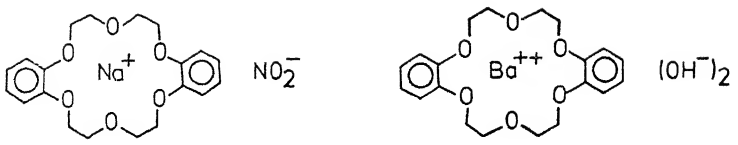
The synthesis and application of various anionic ligands is of great importance for developing the chemistry of synthetic macrocyclic complexones and their analogues.

Several inorganic compounds with the "crowned heavy" cations are of special interest as well. These substances possess particular characteristics, which are, as a rule, liposoluble and it is one of the most important objects of modern chemistry of synthetic macrocyclic complexones and their analogues to study their compounds.

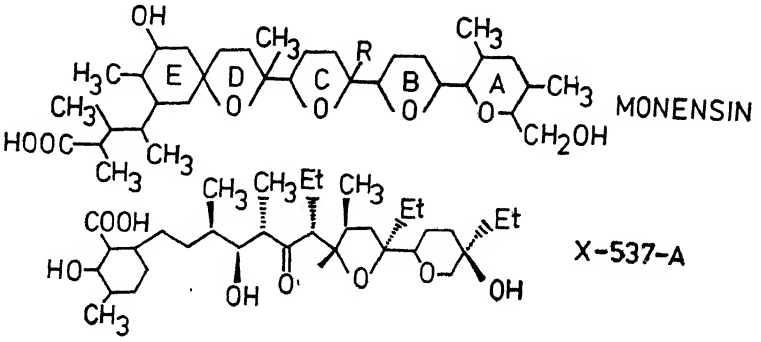
It concerns also the salts described by J. M. Lehn; here are both heavy cations of alkali metal and its anion in them (Lehn, 1977).



We have synthesized a series of some compounds consisting of amorphous and crystalline composition useful for chemical, crystallographical and other investigations.

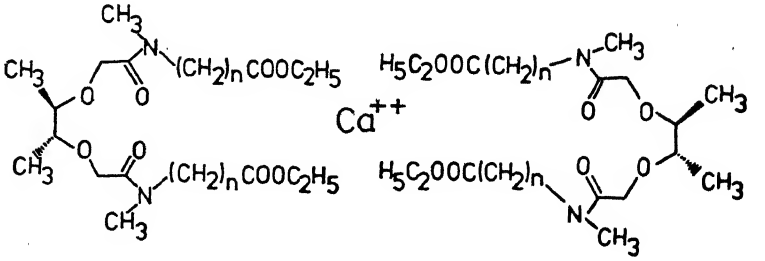


Among the natural ionophores, there were found not only macrocyclic compounds (valinomycin, ennatin, etc.) but also some noncyclic compounds to form pseudomacrocycle with the alkaline earth metal ions. These are monensins, X-537A antibiotics.

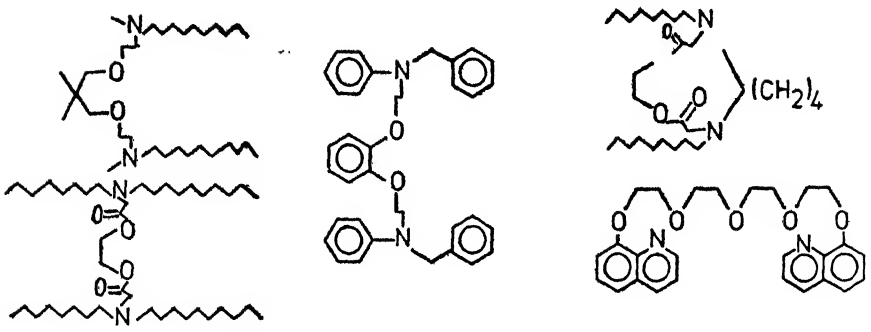


These compounds proved to be peculiar ionophores. Monensin regulates the ion exchange in organisms of animals, sodium and calcium ions especially; X-537A has certain character as for calcium but it is also capable of interacting with cathecholamine taking them out of the organism. A23187 antibiotic also appeared to be an ionophore very specific for calcium ion. It is, used now as the base while making antiinfarction drugs, controlling calcium exchange in cardiac muscle. The synthetic macrocyclic analogues containing long chains and characteristic functional groups are excited by analogues with ionophores such as monensin or A23187.

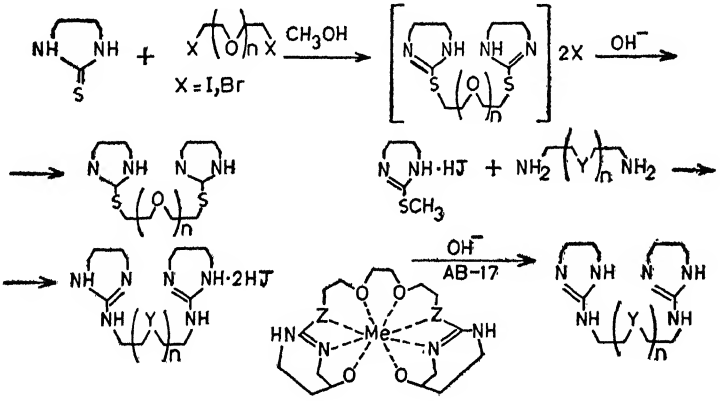
One of the first new synthetic ionophores of non-cyclic type was an ester that proved to be a rather specific ligand to calcium ion (Kirsch *et al.*, 1977).



Then the synthesis of the group of non-cyclic ligands took place, the last like macrocyclic complexones according to their complexing ability (Bissig *et al.*, 1978).



We have also been working in this direction at our Institute. Recently, we have managed to synthesize some substances which are excellent analogues of macrocyclic complexones and their properties are being studied presently.



In recent years, a great success has been achieved in the synthesis of A-23187 antibiotic analogues that have paved the way to synthetic antiinfarction drugs; these ligands are highly specific to formation of complexones with calcium ions and to transfer these ions across biological membranes.

As one can see in Fig. 4, the activity of compounds I, II and III is higher than that of the X-537A antibiotic, and as for the compound II, it approximates to that of A23187 antibiotic, which is considered unsurpassed in the concerned achievements (Wierenga *et al.*, 1979).

Now we are able to conclude that an intensive search for compounds with open chains of carbon and heteroatoms including heterorings and functional groups will help carry ions and molecules across various membranes.

If crown ethers can be considered as analogues of natural ionophores and cryptands and polyhedral macrocycles as their nonadequate likeness, then polyesters synthesized now in quantity and other complexones with the open polyester chains containing heterocycle fragments can be considered as models and similarities of channel-ionconductors. A wide synthetic research in this direction is called for.

Some of the synthetic complexones and their analogues can be used as biologically active substances and drugs (regulation of ion exchange in organism, controlling concentration of catheholamines, etc.). Work in this direction has started with the help of day to day information given by membranology and physicochemical biology.

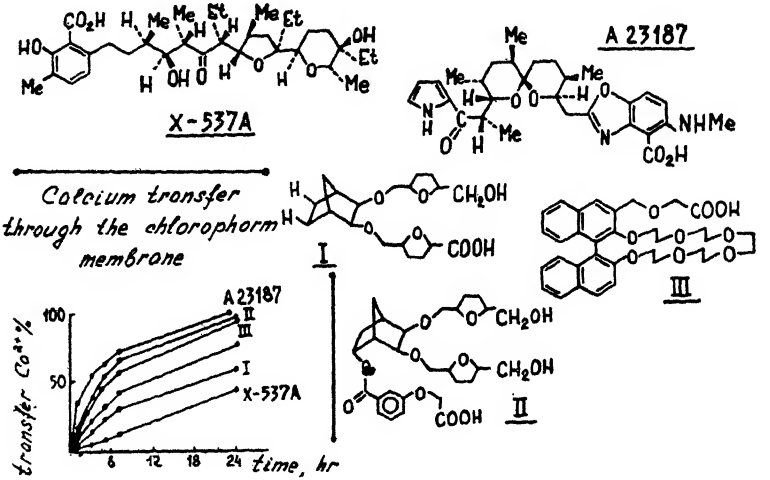
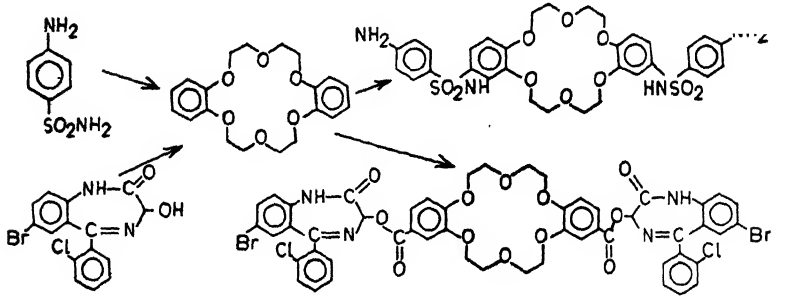


FIG. 4. Synthetic models of X-537 A and A-23187 antibiotics.

Using the synthetic macrocycles it is now possible to make another group of drugs, which we call "containers." The well-known biologically active substances are combined with ionophore fragment by means of quite labile chemical bond. The formula of "container" compounds, which have been synthesized by us, is shown below. Sulphanilamide is one of the most popular drugs. Phenazepam is one of the newest tranquilizers created in the USSR at the Physico-Chemical Institute of the Ukrainian Academy of Sciences.



The essence of these substances is that the ionophore fragment must promote successful penetration of the substances across biological membranes with certain characteristics. Now it is difficult to say how fruitful this idea will be. However,

according to our data "container" sulphanilamide is more active than an ordinary one. These results look promising and give a base to optimism in the area of research.

This paper does not claim to have analysed all the problems of developing the chemistry of the synthetic macrocyclic complexones and their analogues. Its object is to attract attention to the new, rapidly developing branch of chemistry and biology. It also testifies the fruitful influence of ideas and methods of physico-chemical biology upon the progress of chemistry in general, and in particular upon the traditional organic synthesis. It reveals the dialectics between two close branches of science.

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Biogenetics

STUDIES ON ALKALOIDS OF INDIAN MEDICINAL PLANTS

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In view of the special interest, both chemical and physiological, attached to the alkaloids, their study has occupied a position of great vantage since the early days of organic chemistry. The developments, during the last twenty years, in new physico-chemical methods for the identification and structure determination of complex organic molecules only resulted in renewed interest in this important class of compounds albeit in new directions, viz. the study of their biosynthesis and its applications including the development of syntheses by the biogenetic routes.

In our own programme also in the Central Drug Research Institute on the development of drugs in the country, the chemical investigation of alkaloids from a wide variety of plants has received special attention. The highlights of some of the new structures obtained from these by the application of the biogenetic theory, their synthesis as well as their implications in drug research are discussed in the lecture.

Keywords: Drug Research; Biogenesis; Novel Compounds

INTRODUCTION

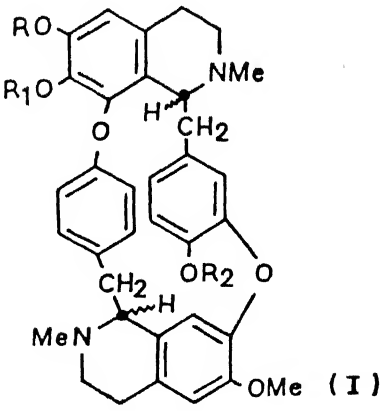
FROM the Ayurvedic formulations since 1000 BC in India to the use of reserpine and vinblastine and their analogues in modern clinical practice has been a long and circuitous route. However, the use of natural products in medicines, even in advanced countries, is quite considerable even to this day. Of the new prescriptions filled in community pharmacies in the USA in 1960, nearly half contained at least one product of natural origin (Scheindlin, 1964).

Amongst the natural products, the alkaloids have always occupied a position of great vantage because of the special interest, both chemical and physiological, attached to their structures. Thus, the researches on the alkaloids from Opium, Cinchona and Strychnos *Nux vomica* make not only fascinating reading but are milestones in the progress of organic chemistry and drug research. In the present lecture, an account is given of some of the interesting alkaloids isolated and studied by our group in the Institute.

Cissampelos pareira

From the roots of *Cissampelos pareira* Linn. (Hindi name: *Zakhme hayata;* fam. Menispermaceae) a number of alkaloids were isolated (Bhattacharji *et al.*, 1956;

and Bhattacharji & Dhar, 1964). These were (*i*) hayatin- (*ii*) hayatinin (*iii*) hayatidin (*iv*) cycleanine (*v*) 1-bebeerine and (*vi*) an orange base named as cissampine. Neither the crude extracts of the plant nor any of these alkaloids showed any anti-tubercular activity for which the plant has a reputation in the folklore of Kashmir. However, hayatin dimethiodide was found to possess pronounced curariform activity (Pradhan *et al.*, 1952; Pradhan & De, 1953, 1959; and Pradhan *et al.*, 1964). These alkaloids were found to have the bisbenzyltetrahydroisoquinoline structure of the type (I).



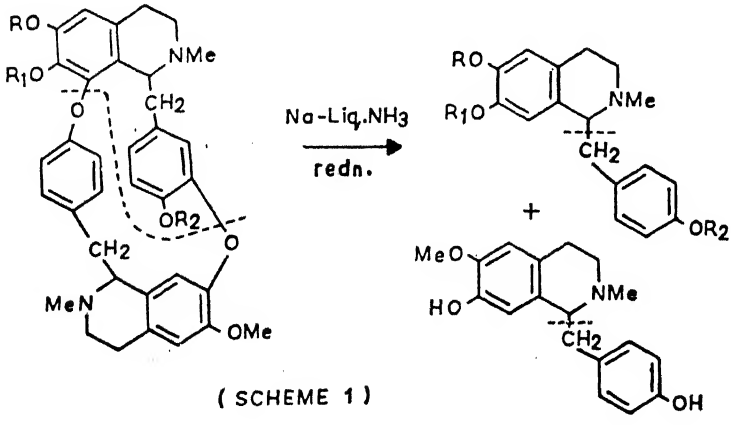
(I)

Hayatinin: dl; R = R_2 = Me; R_1 = H

Hayatin: dl; R = Me; R_1 = R_2 = H

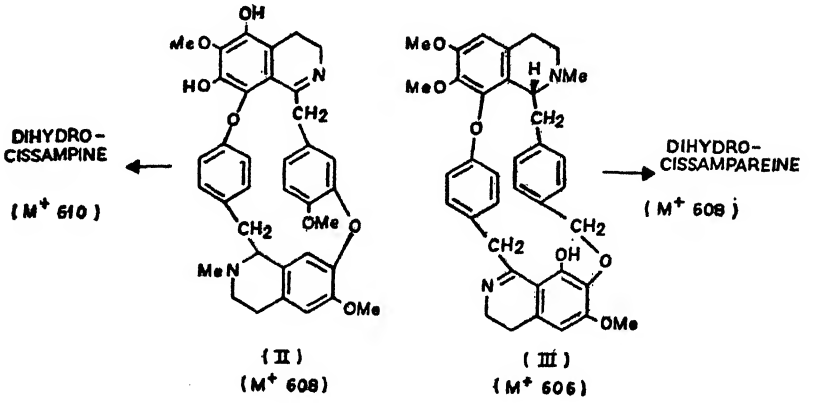
Hayatidin:(S,R); R = R_2 = Me; R_1 = H

Thus the structures of hayatinin (Bhatnagar & Popli, 1967), hayatin (Bhatnagar *et al.*, 1967 *a*) and hayatidin (Bhatnagar *et al.*, 1967 *b*) were established as *dl*-4′ -O-methylbebeerine (I; R = R_2 = Me; R_1 = H), *dl*-bebeerine (I; R = Me; R_1 = R_2 = H) and (S, R)-4′ -O-methylbebeerine (I; R = R_2 = Me; R_1 = H) respectively. This was done by the sodium liquid ammonia reduction of the ethyl ethers of these compounds followed by the characterization of the coclaurine fission products thus obtained (Scheme 1) by spectroscopic methods and subsequently by syntheses.

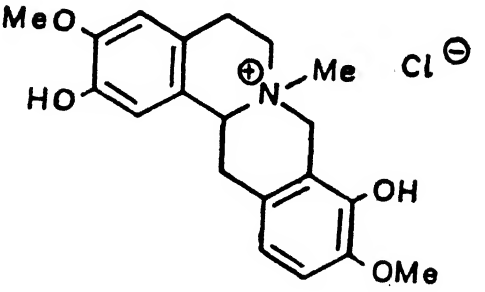


(SCHEME 1)

The fact that hayatin and hayatinin were *dl*-compounds was of considerable biogenetic interest since it indicated that during the formation of these compounds in the plant, the (+)- and (—)-coclaurine units in the pool were getting interconverted by oxidation-reduction *via* the dehydro-coclaurines, prior to their dimerization to give the bisbenzyltetrahydroisoquinolines. That this was indeed the case was shown by tracer studies. When ^{3}H-labelled (+)- and (—)-coclaurine, ^{3}H - and ^{14}C-doubly labelled coclaurines and doubly labelled 1, 2-dehydrococlaurines were fed to *C. pareira,* it was found that these were efficiently incorporated in hayatin (Sharma *et al.,* 1981). These results received further support from the isolation from some collections of the plant of cissampine (II), a red base, in which the upper half was a benzyldihydroisoquinoline and the lower half the normal benzyltetrahydroisoquinoline (Bhatnagar *et al.,* 1967 *c*) Cissampine was thus analogous to cissampareine (III) isolated by Kupchan *et al.* (1966).



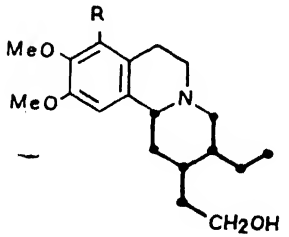
As expected from a plant of this family further work on the water-soluble fractions led to the isolation of quaternary bases (Srivastava & Khare, 1964) of the protoberberine type. The major alkaloid of these, cissamine chloride, was shown (Anwer *et al.,* 1968) to have the structure (IV) and thus found to be identical with cyclanoline chloride isolated earlier from *Stephania tetrandra* by Tomita *et al.* (1967).



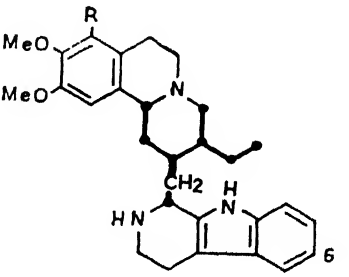
(IV)

Alangium lamarckii

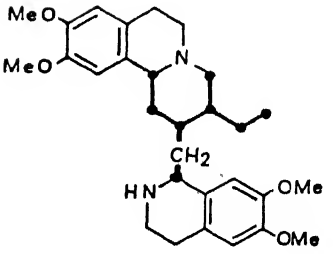
From the leaves of *Alangium lamarckii* Thw. (fam. Alangiaceae) we were able to isolate (Battersby *et al.*, 1966) dihydroprotoemetine (V; R = H) and ankorine (V; R = OH). The occurrence in the plant of these two alkaloids in addition to the known tubulosine (VI; R = H and OH at position 6) and emetine (IX) (Budzikiewiczs *et al.*, 1964) deoxytubulosine (VI; R = H) (Battersby *et al.*, 1965) was of considerable biogenetic interest. It pointed clearly to the fact that a new series of alkaloids with an additional phenolic group (e.g., VI; R = OH) were likely to be found. A search for this compound was thus made and alangimarckine (VI; R = OH) was indeed



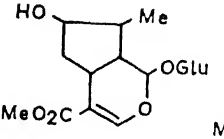
(V); R = H or OH



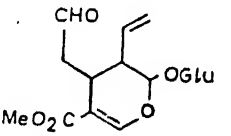
(VI); R = H or OH



(IX)



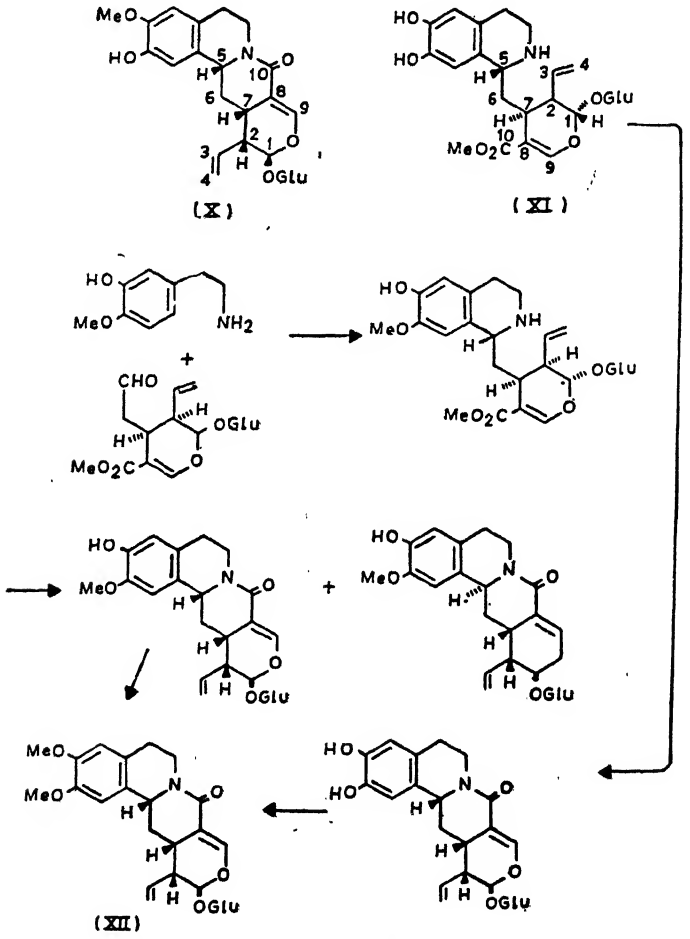
(VII)



(VIII)

found in the leaves of this plant. More interesting results were to follow. All these compounds, it would be noticed, contain a tyrosine derived unit in the top half and a tyrosine or tryptophan derived residue at the bottom with a C_9 unit in between (shown in heavy lines). This was the time when the puzzle of indole and *Ipecacuanha* alkaloid biogenesis was ultimately getting untangled and it was shown by tracer studies that the 'C_9-C_{10}' unit arose out of loganin (VII) and secologanin (VIII) which were the biointermediates in the biogenesis of these alkaloids (Bettersby *et al.*, 1969; and Scott, 1970). It was now obvious that since loganin and secologanin were

involved in the biosynthesis of dihydroprotoemetine, ankorine, and other related alkaloids in *A. lamarckii*, the isolation of monoterpenoid glycosidic alkaloids from this plant would be of considerable interest in view of the reported anticancer activity of emetine (IX). A search, was, therefore, made for new compounds in fractions where glycosides would normally be located and we were gratified to obtain loganic acid as well as new compound, $C_{25}H_{31}NO_{10}$, M^+ 505 which was named as alangiside. Its structure as (X) was established (Kapil *et al.*, 1971) by (i) spectroscopic studies

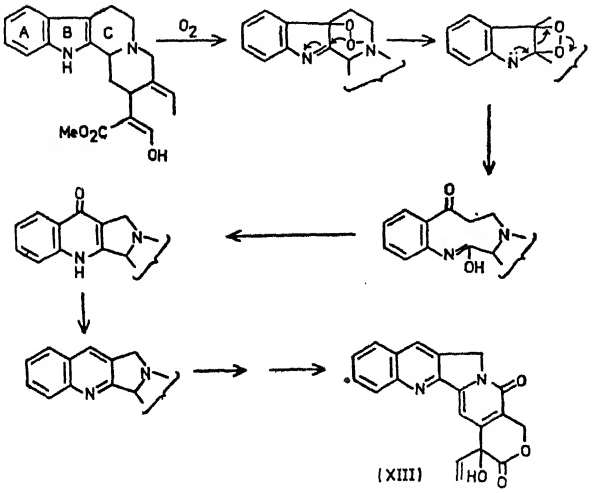


SCHEME 2 : Synthesis of O-Methylalangiside

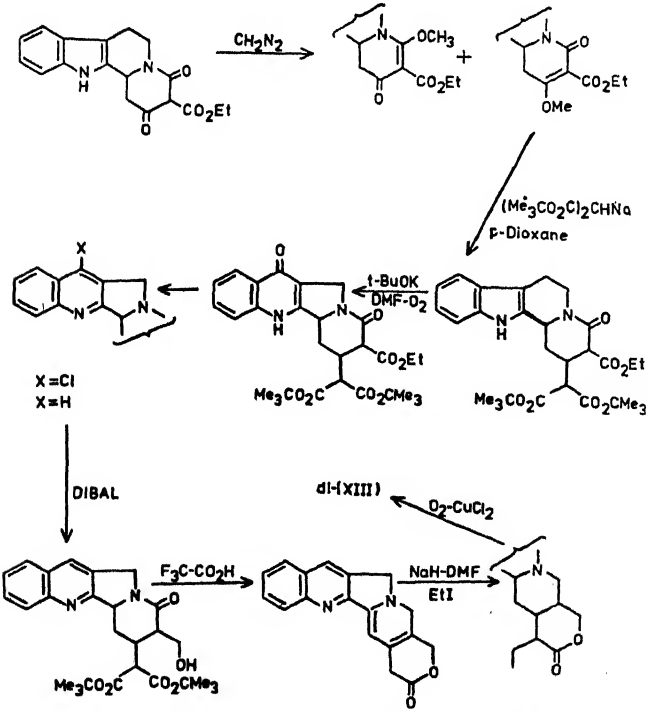
of the compound and its derivatives, (ii) by correlation with desacetylipecoside (XI) and (iii) finally by the synthesis of O-methylalangiside (XII) by the biogenetic route (Scheme 2) (Shoeb *et al.*, 1975).

Mappia foetida

Related to this was some work on the alkaloids of *Mappia foetida* Miers (fam. Icacinaceae). Interest in this plant arose because of the presence in it of camptothecin (XIII), a novel quinoline alkaloid possessing powerful antitumour activity. It was



SCHEME 3 : Wenkert's proposal for biosynthesis of camptothecin.

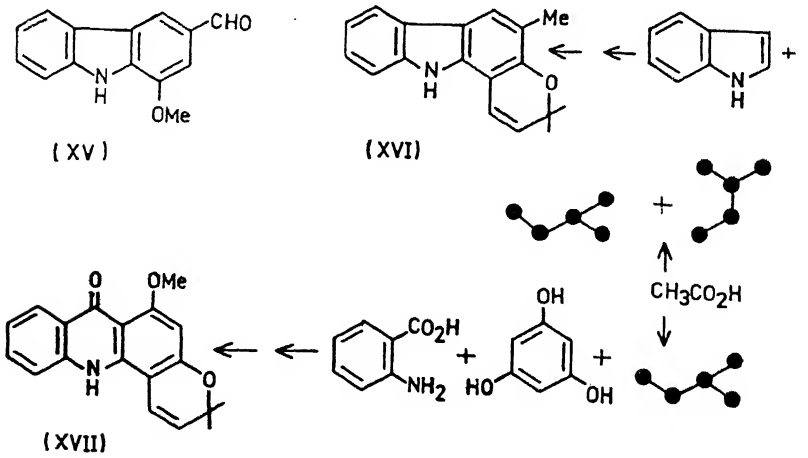


SCHEME 4 : Winterfeldt's synthesis of camptothecin

Wenkert's suggestion (Wenkert *et al.*, 1967) that it was a masked indole alkaloid in which, by the scheme depicted below (Scheme 3), the ring (B) of the indole moiety had got contracted with concommitent shrinking of the ring (C). Feeding experiments (Popli & Scott, 1972; and Hutchinson *et al.*, 1974) with (i) [aryl-^{3}H] tryptophan, (ii) [aryl-^{3}H] tryptamine, (iii) *dl*-tryptophan-3-^{14}C (side chain label), (iv) 6-^{3}H-secologanin, (v) aryl-^{3}H-geissochizine, (vi) aryl-^{3}H-vincoside-isovincoside mixture and (vii) aryl-^{3}H-vincoside lactam showed that Wenkert's original idea of camptothecin being a masked indole alkaloid was correct. However, geissochizine (XIV) was not a precursor nor could its presence in the plant be detected by radio-active dilution technique. Vincoside lactam also was not incorporated. Later work by Hutchinson and Wenkert (1974) showed that isovincoside lactam was the real precursor. Based on these ideas, Winterfeldt was successful in the synthesis of camptothecin by the biogenetic route (Scheme 4) (Winterfeldt *et al.*, 1972).

Murraya koenigii

We now come to the alkaloids of *Murraya koenigii* Spreng., a plant of the Rutaceae family. It is found all over India and its leaves are widely used for imparting flavour to curries. The plant is rich in carbazole alkaloids; the first in these series, girinimbine



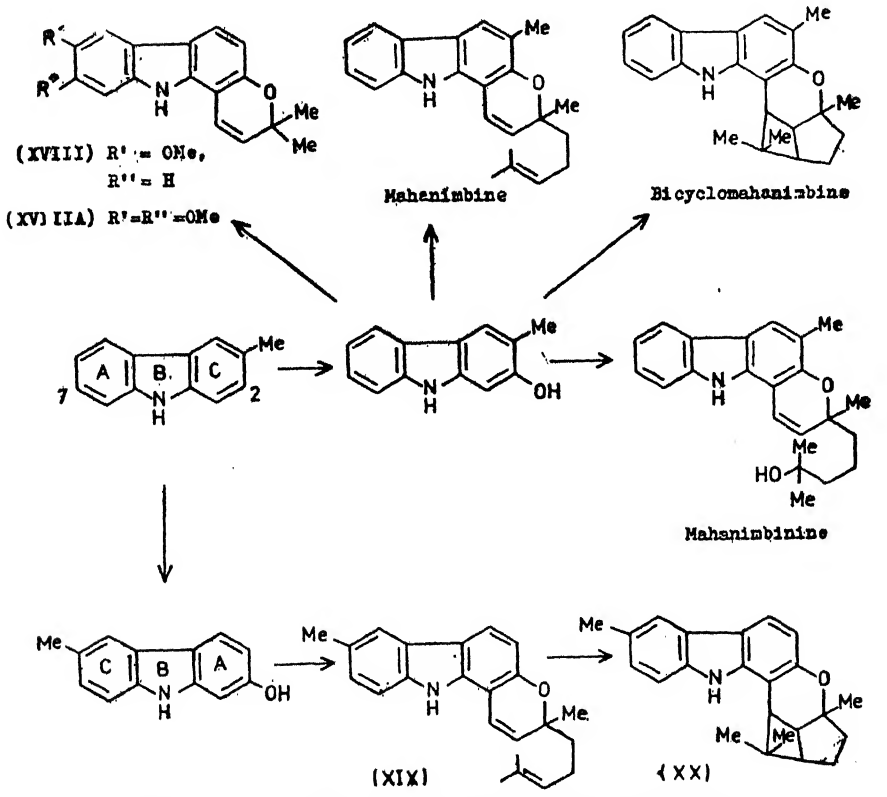
SCHEME 5 : Building blocks for girinimbine and acronycine

(XVI) and murrayanine (XV) were reported by Chakraborty and coworkers (1964, 1965). Our interest in it arose because of the:

(i) similarity of the building blocks (Scheme 5) for girinimbine and acronycine (XVII), a powerful anticancer compound obtained from *Acronychia baueri* Schott. and

(ii) to study the origin of the 3-methyl group in girinimbine.

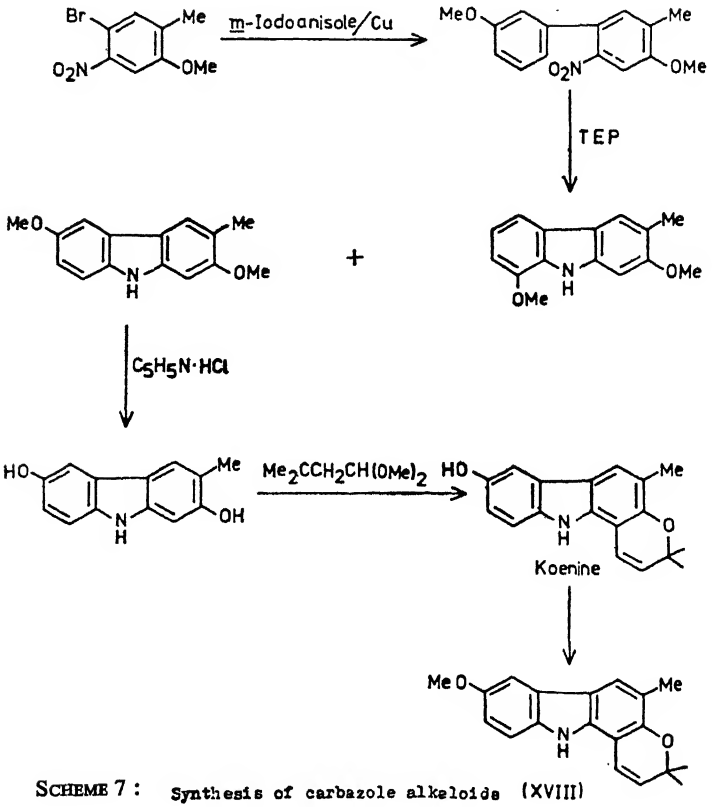
Very soon koenimbine (XVIII) and koenigicine (XVIIIA) were isolated from *M. koenigii* (Kureel *et al.*, 1969 *a*, *b*) and tracer experiments suggested that the 3-methyl group in all these alkaloids was part of a C_5 unit derived from MVA (Kapil & Popli. 1969). Three other groups were also engaged in this work and a number of interesting compounds were obtained (Narasimhan *et al.*, 1968, 1970; Kapil 1971; and Chakraborty 1977). Keeping 3-methylcarbazole in the key position, and further hydroxylation taking place either at the 2-or 7-positions, it was reasonable to visualize (Kureel *et al.*, 1970 *a*) that two series of compounds—one in which



SCHEME 6 : Two series of alkaloids from *Murraya koenigii* Thw.

the methyl and the hydroxyl groups would be on the same side and the other in which these groups would be on the opposite sides—were likely to be produced in the plant (Scheme 6). This indeed turned out to be the case, and the isolation (Kureel *et al.*, 1970 *a*) of mahanimbicine (XIV) and bicyclomahanimbicine (XX)—novel though these compounds were—was not a surprise.

In view of the interest aroused in the new alkaloids, a method for the synthesis of these was devised (Kureel *et al.*, 1969, 1970 *b*). This is illustrated in the Scheme 7 as shown:

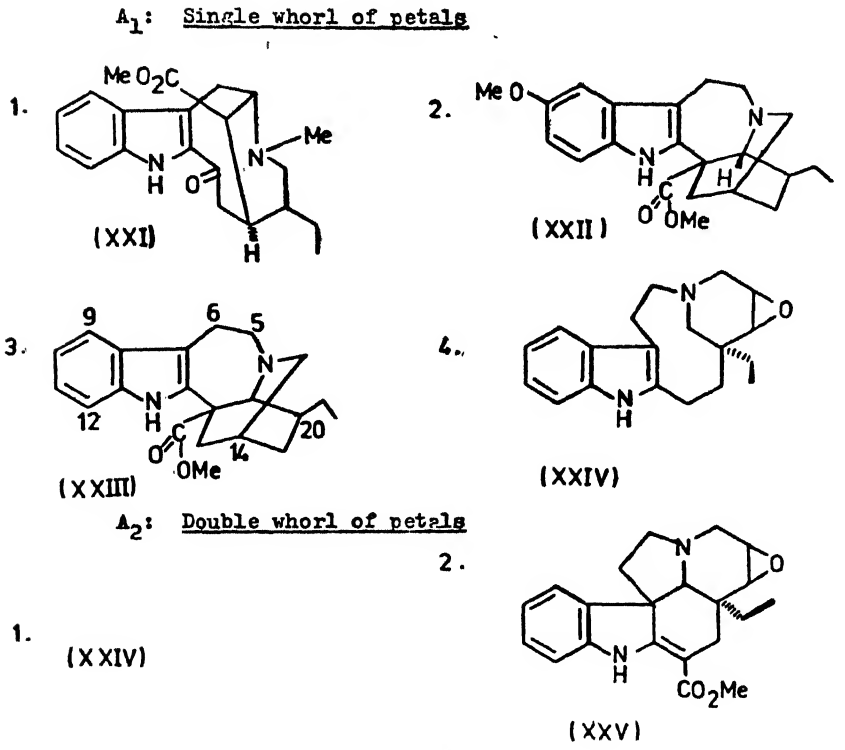


SCHEME 7 : Synthesis of carbazole alkaloids (XVIII)

Tabernaemontana divaricata

Tabernaemontana divaricata R. Br. ex Roem and Schult is grown throughout India in gardens as an ornamental shrub. This species is represented by two varieties; one with petals in a single whorl (var. 1) and the other with petals in two whorls (var. 2). There are marked morphological differences (Raghuvanshi & Chauhan, 1969) between these two and chemical investigations (Raj *et al.*, 1974) on the leaves of this plant showed that var. 1 contained tabernaemontanine (XXI), voacangine (XXII), coronaridine (XXIII) and voaphylline (XXIV) whereas only voaphylline and lochnericine (XXV) were obtained from the leaves of var. 2.

With the isolation of tabernaemontanine, voacangine and voaphylline, *T. divaricata* thus became the seventh instance in the family Apocynaceae which produces all the three main types of indole alkaloids. This, coupled with the reported anticancer activity in the crude extractives of this plant (Hartwell, 1972), prompted



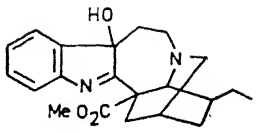
us to examine the roots of this plant as well. Interestingly tabernaemontanine and voaphylline which were isolated in minute quantities and voacangine isolated in good yield from the leaves of this plant could not be detected in the root bark in our investigation (Rastogi *et al.,* 1980 *a*). The chloroform extract of the root bark of var. 1, however, exhibited anticancer activity. This was thus examined in detail and resulted in the isolation of (i) coronaridine, (ii) coronaridine hydroxyindolenine (XXVI), ibogamine (XXVII), 5-hydroxy-6-oxocoronaridine (XXVIII), 5-oxocoronaridine (XXIX), 6-oxocoronaridine (XXX), (±)-19-hydroxy-coronaridine (XXXI), 3-oxocoronaridine (XXXII) and voacamine (XXXIII). The alkaloids (XXVIII), (XXIX), (XXX) and (XXXI) are new compounds, whereas (XXVII) and (XXVI) were reported from this plant for the first time.

B: Roots

1. Coronaridine

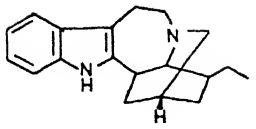
(XXIII)

2.



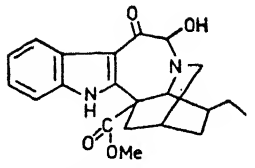
(XXVI)

3.



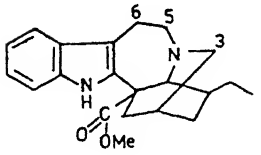
(XXVII)

4.



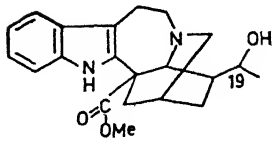
(XXVIII)

5-7



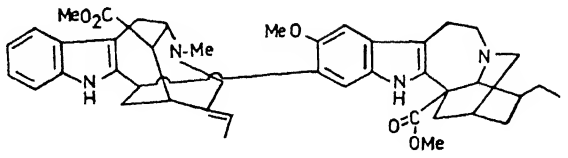
5-Oxo(XXIX); 6-Oxo(XXX) & 3-Oxo(XXXII)-Coronaridine

8.



(XXXI)

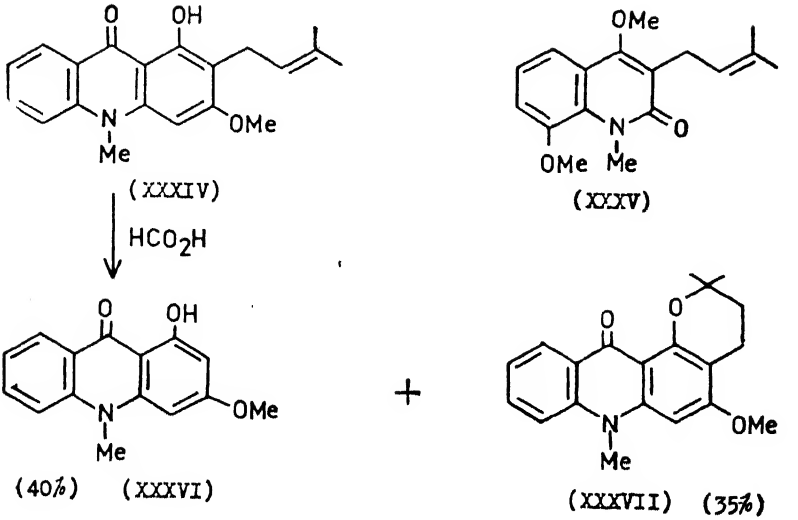
9.



(XXXIII)

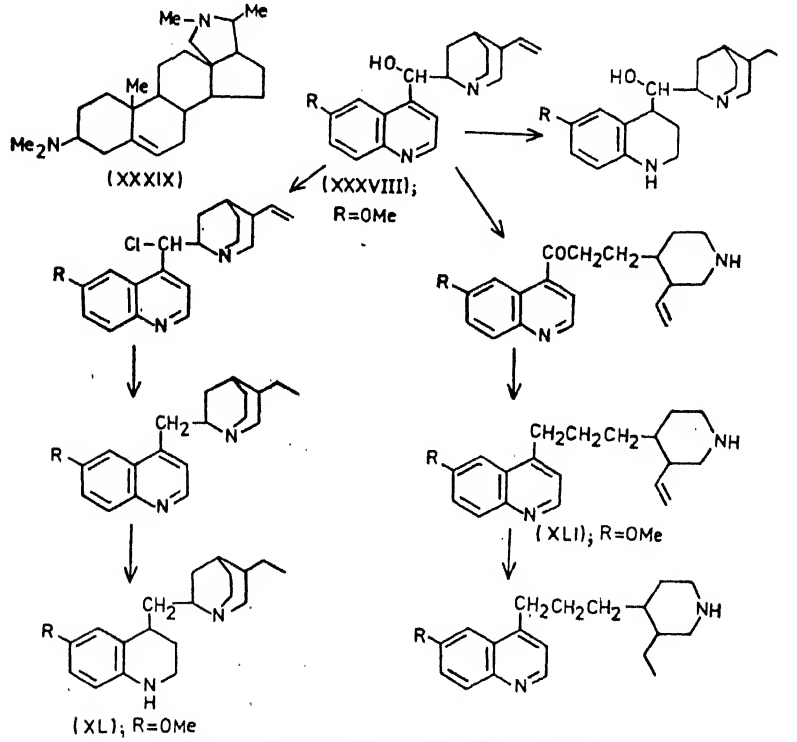
Glycosmis mauritiana

Glycosmis mauritiana (Lam.) Tanaka is a plant of the Rutaceae family and the examination of the roots of this plant led to the isolation (Rastogi *et al.*, 1980 *b*) of glycozoline, glycozolidine, dictamnine, skimmianine, arborinine and two new alkaloids — 1-hydroxy 3-methoxy-2-(3-methylbut-2-enyl) N methylacridan-9-one (XXXIV) and 4,8-dimethoxy-3-(3-methylbut-2-enyl)-N-methyl-2-quinolone (XXXV). The structures of these new bases were established by chemical and spectroscopic methods and confirmed in the case of (XXXV) by synthesis. Interestingly, the formic acid-catalysed cyclisation of (XXXIV) gave the dealkylated product (XXXVI) along with the pyrano-[2, 3-a]-acridone (XXXVII).



Alkaloids as Starting Materials for Syntheses

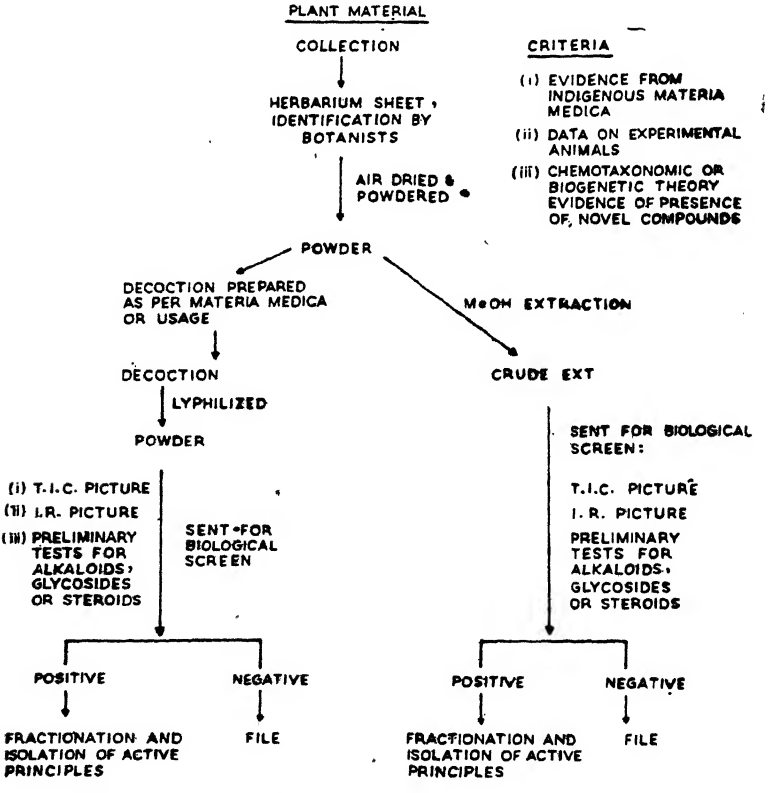
Many a time, the alkaloids obtained in nature are of quite a complex and interesting structure but have found no use as pharmacological agents. Modification of their structures, built up so elaborately by nature, to a chemical profile considered



Scheme 8 : Modification of cinchona alkaloids

responsible for the activity of related compounds, is then a tempting exercise. Many years ago, when malaria was very nearly under control in India and the use of quinine (XXXVIII) was on the decline all over the world, there was a glut of this important compound in the country and there was an urgent need to find alternative uses for the same. This work was then taken up and based on the structures of emetine (IX) and conessine (XXXIX), a number of compounds were prepared (Popli & Dhar, 1959, 1960) by the modification of Cinchona alkaloids (Scheme 8). Two of these compounds, desoxyhexahydroquinine (XL) and α-[3-vinylpiperidyl-(4)]-γ-[6′-methoxyquinolyl-(4′)]-propane (XLI) showed pronounced activity in the experimental amoebiasis in rats (Singh & Sharma, 1959).

This, in a way, is the part of our work that I have tried to present before you. A question may well be asked as to the outcome of all this work in the way of the drugs discovered. Some compounds with pronounced activities have , indeed, been obtained but, so far, the stage has not yet reached when any major breakthrough can be claimed. Drug development from natural products is admittedly a complex exercise involving as it does in many variables. At our Institute, we have been following the screening of plants in experimental animals by the scheme as shown (Scheme 9). We have also started another approach recommended by Dr Sukh Dev (1977). This requires the testing of the crude plant products in the form as suggested in the



SCHEME 9 : Protocol for examination for biological screening.

indigenous system on a clinical pharmacological basis. Hopefully, the two strategies being followed side by side may yield a good drug apart from novel compounds with interesting chemistry.

Acknowledgements

In the work presented, many colleagues have participated. Their names are mentioned in the references. The contribution of Dr R. S. Kapil is especially acknowledged. He participated at every stage of the work from planning to the execution and bore the brunt of day to day problems.

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Medicinal Plants

CHEMISTRY OF SOME BIOLOGICALLY ACTIVE INDIAN MEDICINAL PLANTS

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In this paper, the chemistry of some biologically active Indian Medicinal plants like, *Adhatoda vasica, Commiphora mukul, Cedrus deodora, Tylophora asthmatica, Garcinia mangostana, Clausena pentaphylla, Coleus forskohlii, Curcuma longa, Albizzia lebeck, Aristolochia indica, Acorus calamus, Mucuna prurita, Nerium indicum, Crotalaria* sps., *Celastrus paniculatus, Cicer arietinum, Allium cepa, Boswellia cerrata, Picrorhiza kurrora, Centella asiatica, Embelia ribes, Pueraria* sps., *Gossipium* sps., *Psoralea corylifolia, Heracleum candicans, Plumbago zeylanica,* etc., *and Rutin* groups *and Silimarin* groups of plants are discussed in detail, with respect to their syntheses and photosensitization. The structures of several chemical products are also shown and discussed.

Keywords: Medicinal Plants; Synthesis; Photosensitization.

INTRODUCTION

THE treatment of diseases by herbal based preparations was the mainstay of the therapeutic arsenal until the nineteenth century. This aspect gradually gave way to active constituents of medicinal plants which had a specific or precise pharmacological activity. During and after the Second World War, a large number of synthetic drugs and antibiotics came into the picture and drugs from the plants came to be looked upon as something outmoded and unfashionable. During this change even those medicinal plants which had great merit in the therapeutic efficacy were forgotten. India had a particularly rich heritage of usage of medicinal plants in its Ayurvedic and Unani systems of medicine besides use of many plants in folk remedies. To some extent, the major population of India relies heavily on the use of herbal remedies for treatment of diseases.

While India adopted the allopathic system of medicine in its cities and towns, the indigenous system of medicine survived due to political and governmental support. This factor ensured the continued presence and usage of herbal products on the Indian scene. One other factor that was responsible for the continued interest on plant drugs among the scientific community particularly the organic chemists and pharmacologists, was the non-availability of petrochemicals at a reasonable price, which in turn led to the scarcity of synthetic basic chemicals and reagents for the development of synthetic drugs, with the result that it was always convenient for a chemist or a pharmacologist to lay his hands on plant products and satisfy his

scientific curiosity. An active interest in the chemistry and pharmacology of plant drugs which started in the early fifties has been gaining momentum, which is evident from the number of papers published in chemistry and pharmacology journals and through work presented in conferences and symposia. Another evidence of this trend is the appearance on the Indian market of a large number of herbal based therapeutic agents which have become very popular within a short time after their launching. To some extent this reversal to Indian herbal products has become a worldwide phenomenon mainly for two reasons. Firstly, no major new leads are coming up from research on synthetic therapeutic agents and antibiotics. Secondly, many of the antibiotics and other synthetic drugs have shown sensitization reactions and other undesirable side effects and there is a feeling that natural products are safer because they are more in harmony with the biological system.

There are several centres of natural product chemistry and natural product pharmacology in India. Some of the scientific centres are located in Bombay, Pune, Jammu, Lucknow, Delhi, Calcutta, Banaras, Bangalore and Madras. This plenary lecture will mainly confine to the chemistry of two classes of plant drugs. Firstly, those drugs whose chemical components responsible for biological activity have been well characterized and identified and from which it is possible to produce standardized drugs acceptable to modern system of medicine. The second category is of plants that show significant biological activity as confirmed by clinical trials, yet the activity has not so far been assigned to any single component of known structure. It is, of course, understood that in this paper only those plants have been chosen, which possess significant biological activity.

Medicinal Plants

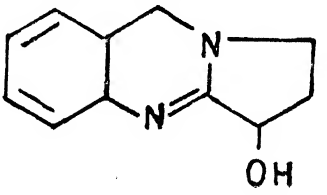
Adhatoda vasica

वासायां विद्यमानायां आशायां जीवित स्यच
रक्तपित्ती, क्षपी, क्रासी किमर्थं अवसीदति।

In this śloka three types of biological activities have been attributed to Vasaka. (*i*) *Raktpita*—an anyurvedic term associated with haemorrhagic disorders most of which belong to the category of capillary bleedings; (*ii*) respiratory diseases like asthma and bronchitis; and (*iii*) control of parasites. A fourth use not described in this śloka but claimed in the indigenous system refers to the use of this plant at childbirth.

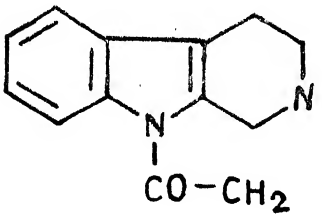
A group of workers at Regional Research Laboratory, Jammu has been able to verify all these claims to be scientifically valid.

Interest in the chemistry of Vasaka originated with Hooper(1888). Ghosh and Sen (1925) isolated a solid compound vasicine which was assigned the structure:



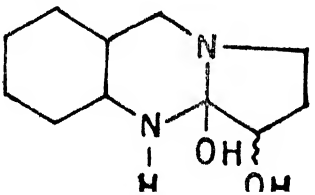
From the roots of *A. vasica* two new alkaloids have been isolated and characterized by the scientists of RRL, Jammu (1980).

Adhatonine (Atal *et al.*, 1980)—a Non-hormonal alkaloid



A—acetamido-3, 4 dihydro pyrido (———4b) indole

Vasicol (Atal *et al., In Press*)



1, 2, 3, 4, 9, 11 hexahydro pyrrollo (2, 1–6) quinazolin—3–11 diol

Apart from vasicine and two new alkaloids, the other reported alkaloids from the plant are: 1. Deoxy Vasicine; 2. Vasicinone; 3. Adhatodine; 4. Anisotine; 5. Vasicolinone; 6. Vasicoline; and 7. Vasicinol.

Vasicine shows marked bronchodilatory activity besides being uterotonic. As such, a number of compounds have been synthesised to find a relation between the structure and the bronchodilatory activity.

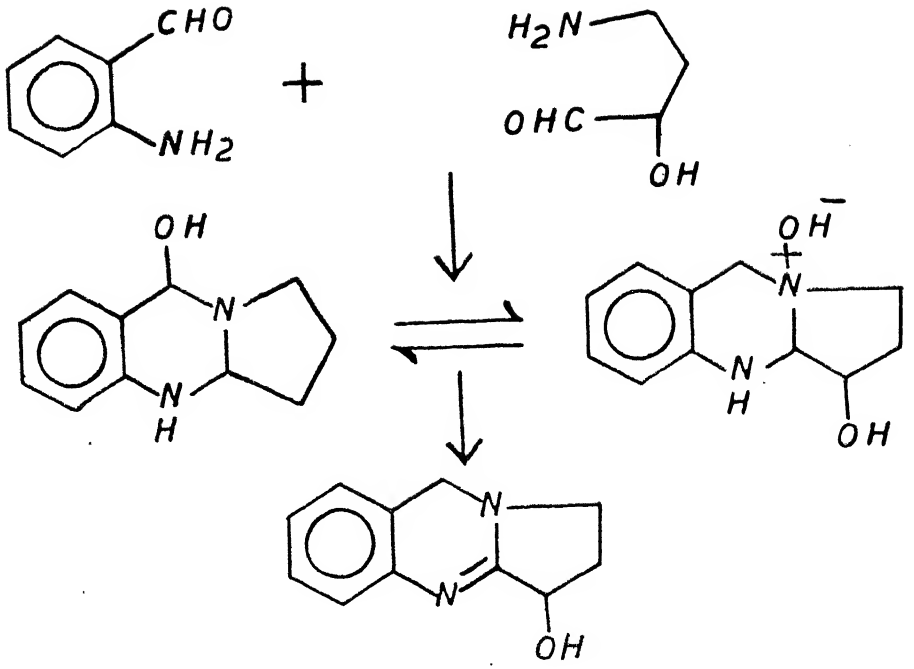
In the first instance, ring A substituted vasicines were synthesized. The procedure for synthesis was based upon Schopf-Oechler Scheme on page 102.

The pharmacological screening revealed no enhancement of activity in the compounds synthesized except for 5, 6-methylenedioxy vasicine which showed uterotonic and bronchodilatory activity, just comparable to that of vasicine. In view of these studies no further substitution of ring A was attempted as no enhancement of activity could be thus speculated.

Our next attention was concentrated on ring C. Deoxy vasicine was synthesized as such by the sequence given on page 102.

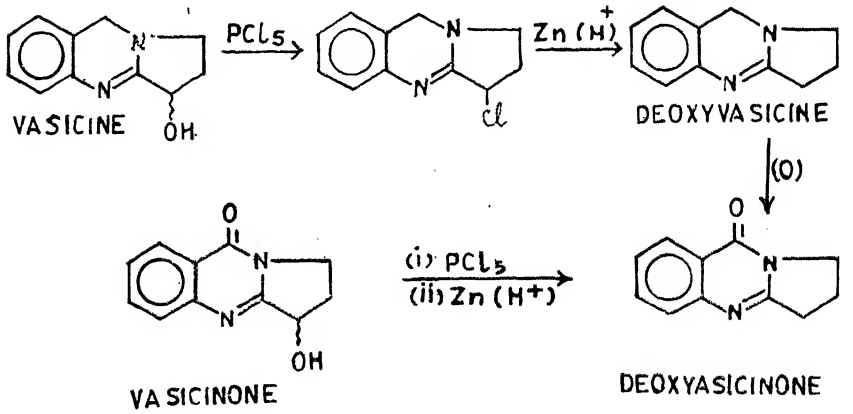
Deoxy vasicine retained some uterotonic activity while it showed only light bronchodilatory activity. This is an indication that the presence of oxygen function at C_3 is necessary for bronchodilatory activity while its presence is not necessary for uterotonic activity.

To establish the role of oxygen function at C-9, vasicinone which has an extra function at C-9 as C=0 was prepared. It was found to be devoid of any activity. Probably two oxygen functions are antagonistic to each other, thus preventing the capacity of molecule to reach the binding site. These studies made us feel that only one of the



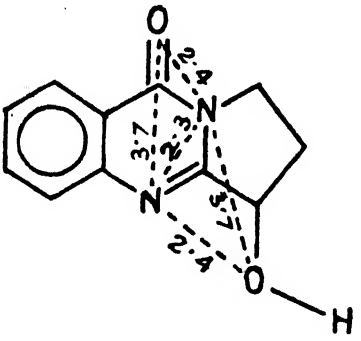
Schopf-Oechler Scheme

1. $R_1=R_2=R_3=R_4=H$
2. $R_1=R_4=H$ $R_2, R_3=$ 
3. $R_1=R_4=H$ $R_2=R_3=O\,CH_3$
4. $R_1=R_2=O\,CH_3$
5. $R_1=O\,CH_3$ $R_2=OH$ $R_3=R_4=H$
6. $R_1=R_2=R_3=O\,CH_3$ $R_4=H$



two oxygen functions is required i.e., either at C-9 or C-3. With this idea deoxy vasicinone was prepared. It showed a little enhancement of activity in broncholdilatory tests thus confirming the need of only one oxygen function either at C-9 or C-3 for

bronchodilatory activity. It was thus visualized that the necessary condition for bronchodilatory activity is N-N-O triangle because in deoxy vasicinone and vasicine identical triangles with same bond lengths are formed.



Bond distance (Å) in N-N-O triangle

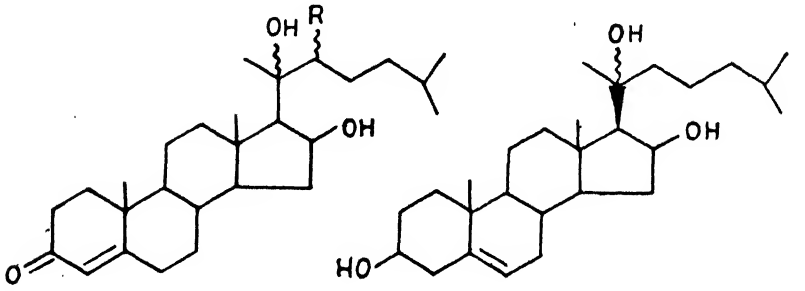
To study the role of ring C i.e., whether the ring size affects the activity or not. Five, six, seven and eight membered ring C compounds were prepared. It was interesting to note that with the increase in ring C size the activity gets enhanced and deoxyhomo C vasicinone was found to be 6–10 times more potent than even aminophylline.

Armed with this data several analogues of vasicine were prepared and SAR studies were carried out.

Commiphora mukul

In Ayurvedic literature the word *guggulu* has been used to cover gum resin obtained from *C. mukul* and has been considered useful for the treatment of rheumatoid arthritis, obesity and allied disorders. Studies at C.D.R.I. have obtained fractions having anti-inflammatory and hypolipidemic activity (Atal *et al.*, 1979).

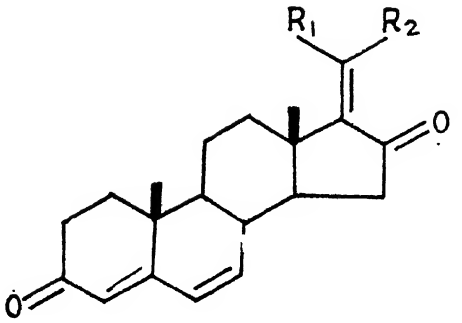
Chemical constituents reported include steroids, diterpenoids, aliphatic esters, and carbohydrates; also minor amount of sesamin and some unidentified constituents. Steroidal constituents include cholesterol and three new sterols—Guggulsterols I, II & III (Sukhdev *et al.*, 1972).



I (R = OH)
III (R = H)

II

From the pet. ether fraction two C_{21} steroids E and Z guggulosterone have been isolated (Patil *et al.*, 1972).

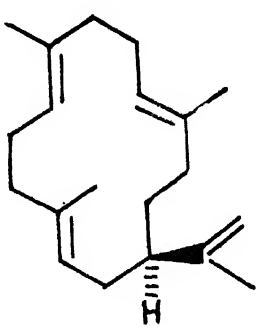


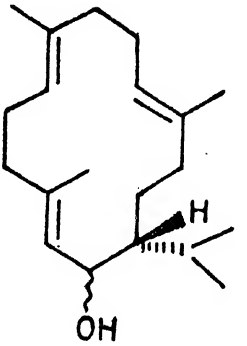
E) $R_1 = CH_3\ R_2 = H$

Z) $R_1 = H\ R_2 = CH_3$

4, 17(20) Pregnadiene-3, 16 dione

The presence of two new diterpenoids Combrene A and mukulol has also been reported (Sukhdev *et al.*, 1973). Combrene A is a tetra-ene derived from geranyl-geranyl pyrophosphate by C_1-C_{14} cyclization.



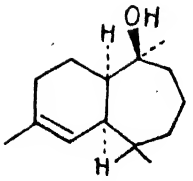


E & Z guggulosterone possess lipolytic and anticholesteremic activities similar to clofibrate. These sterols also control the ADP, adrenalin and serotonin induced aggregation of thrombocytes in a manner similar to clofibrate.

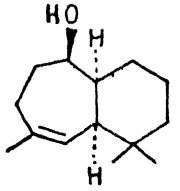
Cedrus deodara

C.D.R.I. during their screening programme had reported spasmolytic activity in the 50 per cent ethanolic/alcoholic extract of *C. deodara*.

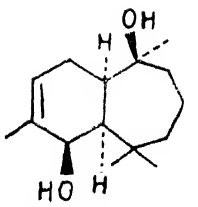
The compounds to which activity has been attributed have since been identified and characterized.



Himachallol



Allohimachallol



Centdarol

All the three are bicyclic sesquiterpenes. Himachallol (Bisarya & Sukhdev, 1968) has only one OH function whereas Centdarol (Kulshreshtha & Rastogi, 1975) is a diol.

Spasmolytic activity is of a non-specific character but it is similar to Papavarins (isolated guinea-pig iluem). This is the first ever report of antispasmodic sesquiterpene.

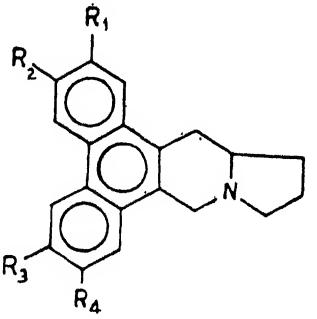
Tylophora asthmatica (*Tylophora indica*)

Leaves of *Tylophora* are used widely for the treatment of asthma. The careful clinical studies of Shivpuri *et al.*(1969) using not only leaves, but also total alkaloids and pure tylophorine have shown to provide relief to the patients suffering from allergic rhinitis and/or asthma.

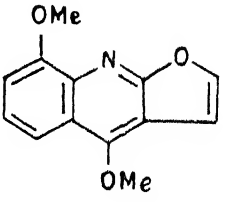
The phenanthro indolizidine alkaloids and furoquinoline alkaloids have been isolated from *Tylophora* sps.

Tylophorine (Govindachari *et al.*, 1974), tylophorinidine, anhydrodehydrotylophorinidine and anhydrodehydrotylophoridine belong to phenathroindolizidine group.

Similar alkaloids have also been isolated from *Cynanchum vincetoxicum* and *Ficus septica*. Fagarine (Shivpuri *et al.*,, 1969) and Skimmanine are of furoquinoline type.



$R_1=R_2=R_3=R_4=OMe$.
TYLOPHORINE

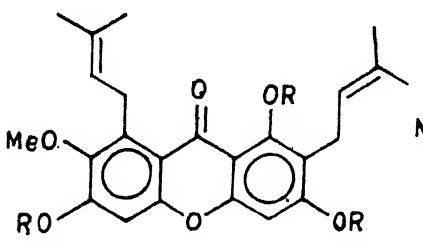


γ – FAGARINE

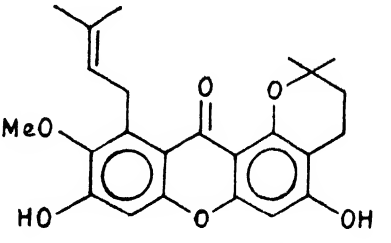
Tylophorine has stimulating action on the muscles of blood vessels (Chopra *et al.*, 1937). Detailed mechanism of action of tylophorine has recently been reported by Lalita Kameshwaram, Madras.

Garcinia mangostana

The fruit peel of *G. mangostana* has been used in India for diarrohea and dysentry. Earlier, only the pharmacology of Mangiferin was reported but other Xanthone glycosides were not investigated (Stout *et al.,* 1968). Now three new compounds have been isolated and characterized (Hostetlmann & Wagner, 1977).



Mangostin (R=H)
Mangostin-triacetate (R=Ac)



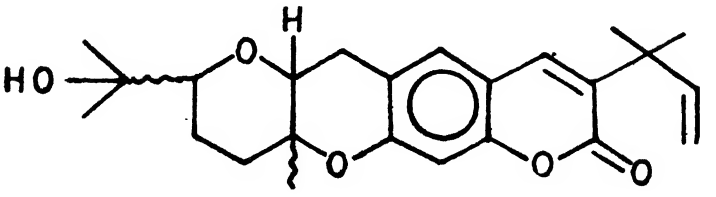
1-isomangostin

All the three compounds have shown a CNS depressant action characterized by ptosis, sedation and inhibition of motor activity and potentiation of phenobarbitone sleep time.

Anti-inflammatory activity was comparable to 1 mg/kg of Dexamethasone, 100 mg/kg Phenylbutazone in experimental models with carragenin induced inflammation and cotton pellet implants (Shankaranarayan *et al.,* 1979). The most interesting feature of these compounds, acting as anti-inflammatory, is that they also possess anti-ulcer effect.

Clausena pentaphylla

Workers at C.D.R.I. have isolated two isomeric terpene coumarins—Clausmarin A & B having marked spasmolytic activity (Shoeb *et al.,* 1978). The structure has been established by C_{13}NMR.



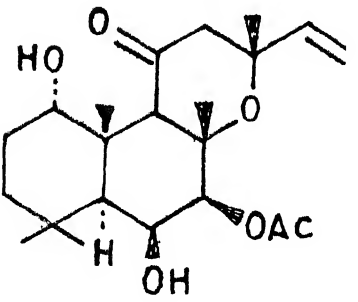
CLAUSMARINE A & B

The novel structure of the compound provides scope for interesting studies.

Coleus forskohlii

Workers at C.D.R.I. have extracted a hypotensive substance from the roots of

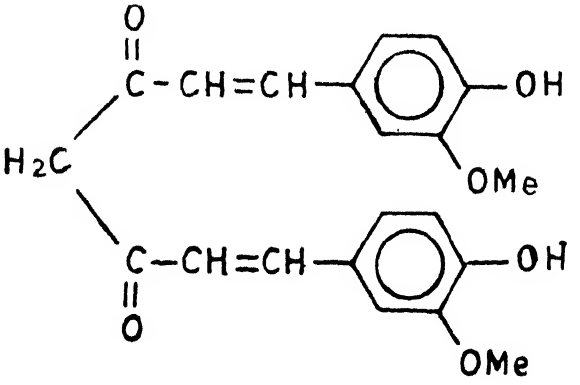
C. forskohlii. The compound has been isolated and characterized as Coleonol (Tandon *et al.*, 1977).



Simultaneously M/s. Hoechst have also isolated a diterpene from the same plant and named it Forskolin (Bhat *et al.*, 1977). Coleonol and Forskolin have been shown to be identical.

Curcuma longa

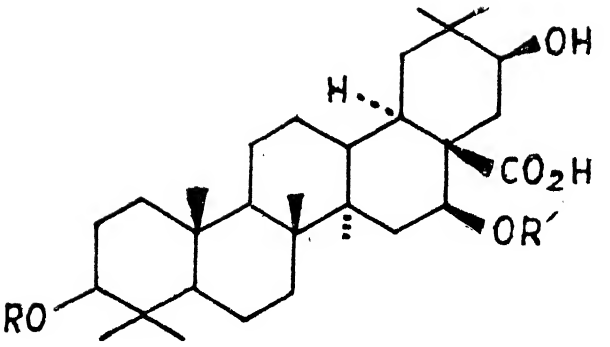
Curcumin is a major constituent of *C. longa.* It is known to possess local as well as systemic anti-inflammatory properties. The anti-inflammatory property of curcumin was evaluated by several models and compared favourably with established agents. (Shukla *et al.*, 1969, 1971).



CURCUMIN

Albizzia lebeck

A. lebeck is a renowned remedy for allergic diseases like bronchial asthma, urticaria and insect bites. Chemical constituents reported from *Albizzia* are acacic acid, albigenic acid (triterpene acids) and albigen, beta-amyrin, alpha-amyrin, lupeol and benzylbenzoate, friedelan-3-one and saponins (lebbekanins A, B, C, D, E) (Varshney *et al.*, 1976).

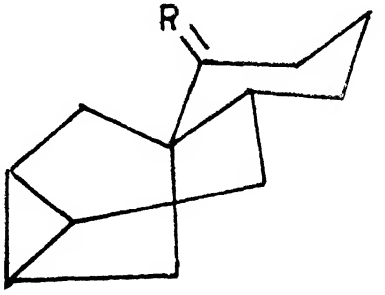


R, R' = SUGARS

Tripathi and Shukla in a study in guinea-pigs have shown the protective action of *A. lebeck* on adrenals against histamine and have suggested that it may be useful for the treatment of bronchial asthma and other allergic disorders.

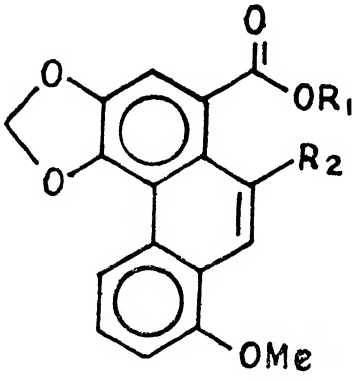
Aristolochia indica

Chemical constituents reported include ishwarone (first) tetracyclic sesquiterpene of eremorphilane type isolated from nature (Govindachari *et al.*, 1970).



Ishwarane H=H_2
Ishwarone R=O
Ishwarol R=C(H)(OH)

And aristolochic acids, I, II, III (Pakrashi *et al.*, 1977).



Aristolic acid $R_1=R_2=H$
Methyl ester $R_1=CH_3$ $R_2=OH$
Aristolochic acid $R_1=H$ $R_2=NO_2$

Pakrashi and co-workers from the Indian Institute of Experimental Medicine have reported the use of desnitro acids and its methyl ester as emmanagouge and abortifacient (Pakrashi *et al.*, 1976, 1977; and Pakrashi & Sharma, 1978).

At RRL, Jammu, we started work on *Aristolochia bracteata* which has been used for the control of insects in some parts of India. Aristolochic acid isolated from *A. bracteata* showed marked larvicidal and chemosterilant activity in four species of insects.

Four synthetically modified derivatives of aristolochic acid were prepared and tested on a comparative basis. It was observed that presence of N function at position 10 and methylene dioxy ring are essential for the activity.

Acorus calamus

Cis-asarone directly affects the gonads of insects and produces sterility in a variety of insects (Atal *et al.*, 1971). Work at RRL Jammu has established that it interferes with: (*i*) the yolk deposition in ovaries; and (*ii*) yolk protein synthesis. Reported constituents include alpha and beta asarones (trans and cis- 1, 2, 4-trimethoxy-5 (1-propenyl) benzene). Beta asarone has been established to be the active insecticidal principal. In addition, sesquiterpenes of cadcilene type and selinam type have been reported. Recently, a sesquiterpene (—) cadala—1, 4, 9, triene has been isolated [*see Phytochem.*, **18**(2), 1979].

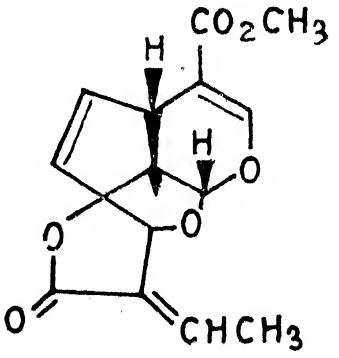
Mucuna prurita

Seeds of this plant have been used in the treatment of nervous disorders since ancient times. It was shown that those seeds contain substantial percentage of L-DOPA (Damodaran & Rangaswamy, 1937). In clinical trials on patients with Parkinson's disease a significant response was obtained without side effects. The therapeutic response could not be ascribed to L-DOPA alone (Vaidya *et al., under publication*). Efforts to discover other active ingredients have not succeeded so far.

Nerium indicum

Chatterjee and co workers (1973) have isolated cardiotonic glycosides from the leaves of *N. indicum.*

Other constituents of *Nerium* are ursolinic acid, oleandrin and a crystalline glycoside—Neriodrin. Cardiotonic activity is due to Plumericin.



Plumericin was isolated from *Plumeria* sps. Other cardienolides isolated from *Nerium* are: Oleandrigenin; Gitoxigenin; and Anhydrogitoxigenin.

Crotalaria sps.

Over 100 alkaloids containing Pyrrolizidine nucleus have been isolated from *Crotalaria* sps. Most of them are known to be hepatotoxic and carcinogenic (Pomeroy & Raper, 1972). Some of them can be modified by simple reactions to non-toxic compounds having a whole range of interesting biological activities.

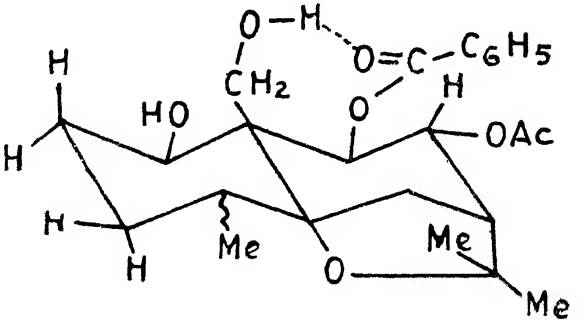
Seeds of *C. retusa* contain as high as 8 per cent alkaloid monocrotaline which provides substantial quantity of natural molecule which can be modified. l-Methylene-pyrrolizidine is another natural molecule available in abundance in *Crotalaria* sps. which can be used for the preparation of analogues. Few examples of semisynthetic modifications (Atal, 1978) are:

1. N-isopropyl-l-methylenepyrrolizidinium bromide — Ganglion blocker
2. N-(p-hydroxyl benzoyl) retronamin — hypotensive
3. Platynecine 7-9-disenecioate — local anaesthetic
4. N-(2′ bromoethyl)-heliotri-danium bromide — muscle relaxant
5. N-(4-phenylphenacyl)-heliotridanium bromide — antispasmodic

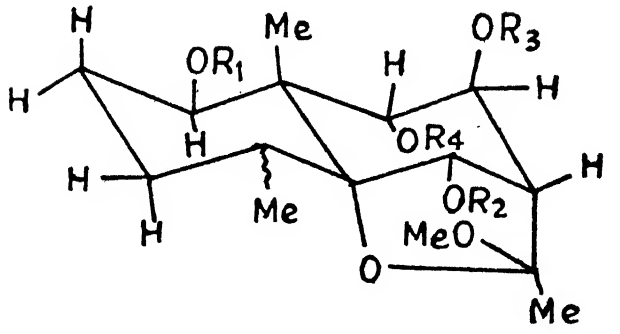
Celastrus paniculatus

Seeds and oil stimulate intellect and sharpen memory (Seth *et al.*, 1963). Aerial parts of the plant were found to possess antiviral activity against Ranikhet disease virus. It has now been conclusively shown that *C. paniculatus* seeds improve the learning process in laboratory animals (Sukhdev *et al.*, 1974, and Wagner *et al.*, 1975). Reported chemical constituents are:

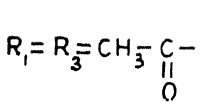
1. Sesquiterpene ester (Kranth, *et al.*, 1980). (Malkanguin-I) (a sesquiterpenetriol—with two hydroxyls esterified with acetic benzoic acids)

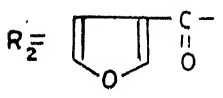


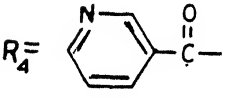
2. Alkaloid—Sesquiterpene-tetraol (Calapanol-II) which is alternately esterified with aceticbenzoic, nictonic and β-furoic acids.



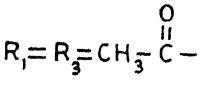
Celapnin

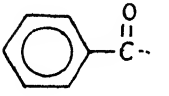


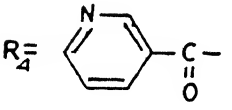




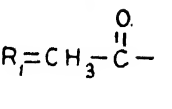
Celapnigin

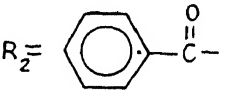


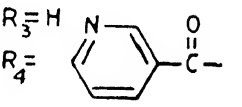




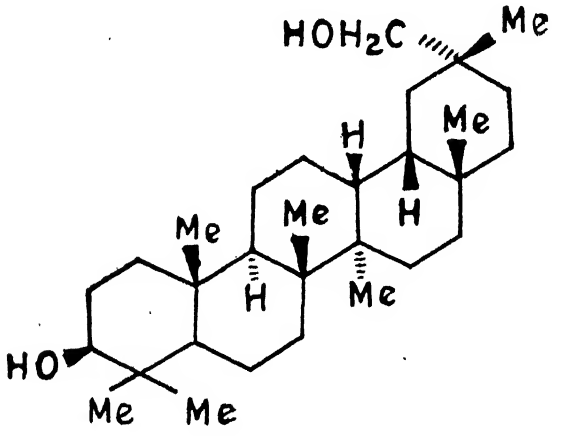
Celapagin







3. A new triterpene diol—Paniculatadiol (Nanavati, 1975).

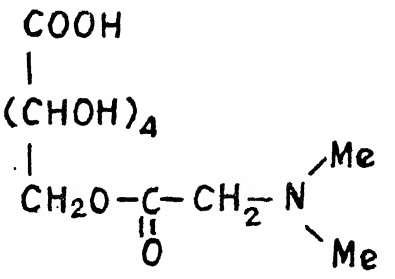


Cicer arietinum

Pangamic acid reported to be present in apricot kernels and various cereals (Krebs *et al.*, 1951), has been widely accepted in many countries as a necessary food factor with important physiological action.

Pangamic acid has been reported to be safe and effective in the treatment of patients suffering from ischemic heart disease associated with cardiopulmonary insufficiency.

It has been claimed to foster a greater adaptation to muscular activity (Telegdy *et al.*, 1969).



Allium cepa

RRL, Jammu have found a variety of *A. cepa* known as red multiplier active for the relief of bronchial asthma. This activity of *A. cepa* is due to Quercetin and Prostaglandin A (detected by radio-immunoassay). In addition to these two compounds, *A. cepa* contains thiols, disulphides, trisulphides, thiosulphinates and an elusive lachrymatory factor (Menon, 1969).

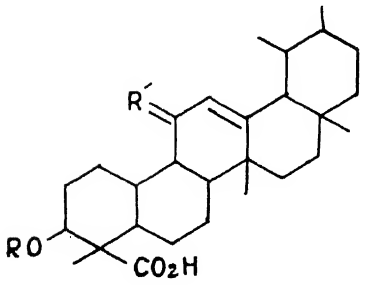
Fibronolytic activity (Augusti *et al.*, 1975) also has been detected in extract of *A. cepa* and has been attributed to:

3, 4-Dimethyl thiophene
Propyl-allyl-disulphide
S-methyl and S-propylcystein sulphoxide

Cycloallin — a sulphur containing amino acids also has shown *in vivo* fibrinolytic activity (Virtanen & Matikkala, 1959) but it has not been possible to demonstrate this activity *in vitro*, suggesting an indirect mechanism.

Boswellia serrata

Guggulu in Ayurvedic literature is applied to gum resin (incense commonly used in household). Guggulu is reputed for the treatment of rheumatoid arthritis and obesity. RRL, Jammu have conducted extensive investigations on many aspects of guggulu and found that gum resin obtained from *B. serrata* is very highly effective for the treatment of rheumatoid arthritis.

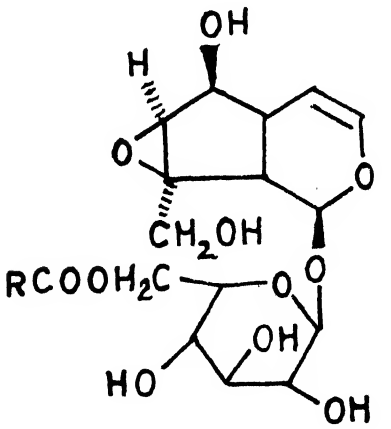


I R = H, R' = H_2
II R = $COCH_3$, R' = H_2
III R = $COCH_3$, R' = O
IV R = H, R' = O

Chemical constituents reported include methyl chavicol, serratol (a diterpene alcohol), amyrins and triterpene acids. Concentrated ethanol fraction has shown marked antiarthritic and anti-inflammatory activity. Bhattacharyya and co-workers (1978) have isolated Boswellic acid (I), Acetyl Boswellic acid, II-keto Boswellic acid and Acetyl-II-keto Boswellic from the total resin. In work conducted on biological activity, it has not been possible to ascribe total activity to any one of the above known constituents. A few uncharacterised but pure compounds show antiarthritic activity of a low order. Therefore, a single highly active principle, if at all present, is still elusive or alternately it is a case where total antiarthritic activity is the result of cumulative synergistic or potentiating action of a number of active compounds.

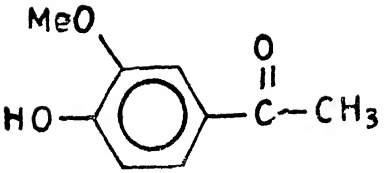
Picrorhiza kurroa

Liver tonic, Liver protecting principles—Picrosides I, II and III (Jastus Liebigs, 1977).



R = Cinnamoyl (I)
R = Vanilloyl (III)
R = 3-methoxy-4-hydroxy cinnamoyl (III)

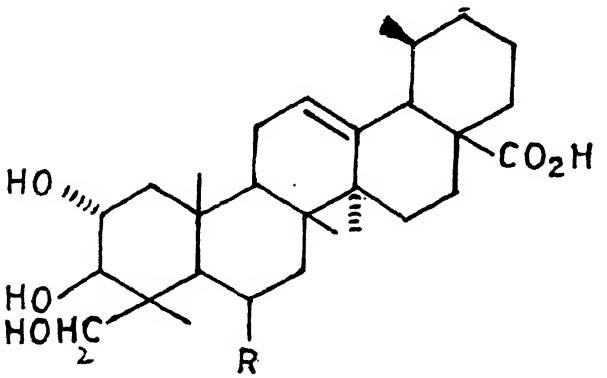
Another constituent—apocyanin possesses (3-methoxy-4'-hydroxy acetophenone) choleretic activity (*See Curr. Sci.*, **40**(22), 1971, 603–604). It doubles bile flow in an anaesthetized dog within one hour after administration and maintains the effect for 3hrs.



Langer and co-workers in a controlled clinical study have observed moderate to marked relief in asthma patients. Remissions of rheumatic pains in 4 cases being treated for asthma were observed.

Centella asiatica

Rastogi and co-workers (1963, 1973) have investigated *C. asiatica* and have reported a number of constituents. Saponins—Brahmoside & Brahminoside (Tri and tetraglycosides of Brahmic acid) Triterpene acids—Brahmic acid, Isobrahmic acid, Asiatic acid, Betulic acid, Indocentoic acid, Madecassic acid.



Asiatic acid R=H
Brahmic acid R=OH

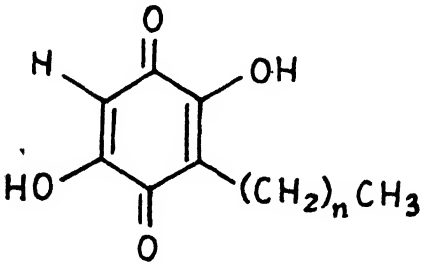
Brahmosides possess sedative action equivalent to minor tranquilizers. Alcoholic extract of entire herb has anti-amoebic activity.

Embelia ribes

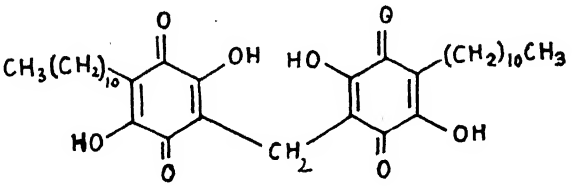
Aqueous extract of the berries of *E. ribes* possesses antifertility activity.

Chemical constituents reported:

Embelin	Homoembelin	Rapanone	Homorapanone
$n=11$	$n=9$	$n=13$	$n=15$



Embelin is not the active principle, because embelin free extracts were equally active (Kholkute *et al.*, 1978). Review of Ayurvedic literature reveals that berries have been used with other plants such as *Piper longum* or *Hibiscus rosasinesis* and *Ferula narthex*. Waltair samples of *E. ribes* contain vilangin (two units of embline with a $-CH_2-$bridge).



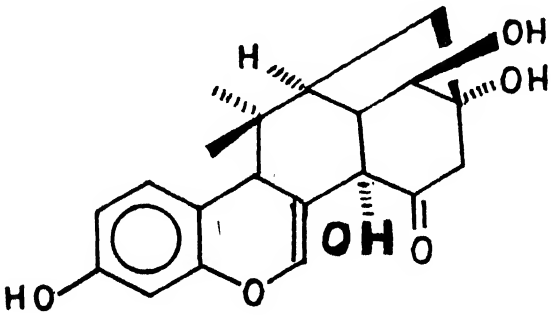
Pueraria sps.

Alcoholic extract of *P. tuberosa* when given to experimental animals has shown estrogenic and progestational activities (*Adam et al.*, 1960).

In clinical trials alcoholic extracts have been found effective for the regulation of menstrual disorders.

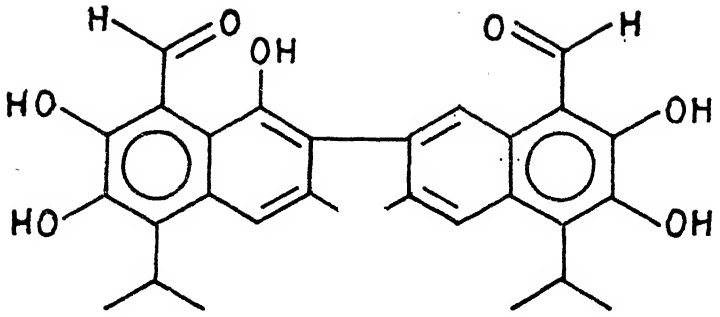
Reported chemical constituents include: Isoflavones and their glycosides (Seshadri *et al.*, 1969) viz., Daidzen Puerarin, Daidzin. However, none of these have been found to be active.

Miroestrol isolated from *P. mirifica* may be the active principle (Scholler *et al.*, 1948). Miroestrol rich fractions prepared from *P. tuberosa* were effective in lower doses.



Gossypium sps.

A biologically active pigment Gossypol has been isolated from cotton seed (*Adam et al.*, 1960). It has 2-2′ bismapthyl structure. It is a natural antioxidant. Antifertility activity of Gossypol has been established in China. A number of Indian workers have confirmed this activity.



Gossypol has also been isolated from *Thesperia populnea* (Berasdi *et al.*, 1969). Its chemistry, stereochemistry and toxicology has been fully investigated (Seshadri *et al.*, 1972).

Furanocoumarins and Photosensitization

Furanocoumarins are a group of substances occurring in nature especially in plants of the families Umbelliferae and Rutaceae. Many furanocoumarins have also been synthesized. Furanocoumarins have a furan ring fused with the 2-H-1-benzopyran-3-one (coumarin) nucleus. The parent member of the linear furanocoumarins is psoralen while that of the angular type is angelicin (isopsoralen).

Furanocoumarins as also other coumarins exhibit a wide variety of biological activity. However, furanocoumarins are especially noted for their photosensitizing effects i.e., the biological effects exerted by them upon irradiation with long wave length UV light.

Some plant extracts and juices increase the photosensitivity of skin. Application of these substances on the skin followed by exposure to sunlight causes erythema and pigmentation. Intense exposure may lead to hyperpigmentation and occasionally vasiculation of the skin. The substances responsible for this action on skin are furanocoumarins present in such plants.

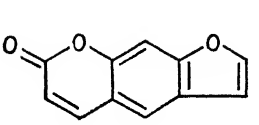
The best known photosensitizing effect of the furanocoumarins is erythema of human or guinea-pig skin, appearing after the application of the substances on the skin followed by exposure to long-wavelength UV light or sunlight. Erythema (oedema and vasicles may also occur) appears after a latent period of several hours, lasts a few days and is followed by dark pigmentation.

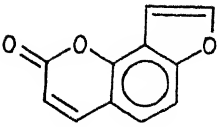
A number of furanocoumarins such as Xanthotoxin (methoxalen, 8-methoxy psoralen), Imperatorin (8-isoprenyl-oxypsoralen) and Trioxalen (2', 4,8-trimethoxy psoralen) are used for the treatment of leucoderma which is characteristic of vitiligo. These furanocoumarins are either given orally or applied locally on the leucodermic spots which are then carefully exposed to sun or long wavelength UV light. In many cases repigmentation is obtained.

Psoralea corylifolia

Seeds of Babchi are used in India for the treatment of leucoderma and other

skin diseases. The curative action of *Psoralea* in leucoderma is due to psoralen and isopsoralen (Angelicin).

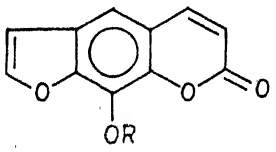


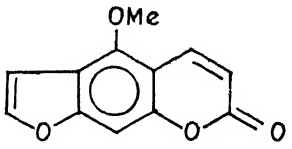


Heracleum sps.

In view of the interesting biological activity exhibited by coumarins a systematic investigation was undertaken at RRL, Jammu. The genus *Heracleum* has about 20 species growing in India, more than half of which grow wild in Himalayan forests. It has been found that the genus *Heracleum* is very rich in furanocoumarins.

Three furanocoumarins viz., Xanthotoxin, Bergapten and Imperatorin were found very effective as photosensitive agents. These three were earlier isolated from *Ammi majus* and used since ancient times in Egypt.





$R = CH_3, R = CH_2-CH=CMe_2$

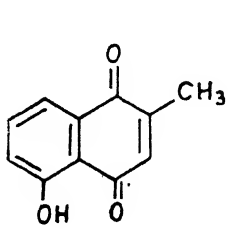
Heracleum candicans gives coumarins majority of which are others of Xanthotoxol.

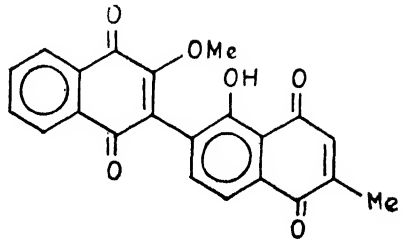
Coumarins isolated from *H. candicans:*
Imperatorin
8-Geranoxypsoralen
Heraclenin
Heraclenol
Xanthotoxin
Bergapten
Sphondin
Isoheraclenin
Ter-O-methyl heraclenol

Plumbago sps.

Plumbago sps. are known for dermal photosensitizing action. This biological activity is mainly characteristic of coumarins, but *Plumbago zeylanica* and *P. rosea* which contain quite a good number of characterised Naphthaquinones have shown this activity in their extracts. *P. zeylanica* has shown better promise as an antileucoderma drug.

An abundant constituent of the species is Plumbagin. Plumbagin-free fraction when concentrated gives the response as a dermal photosensitizing agent. Thus, this activity is not attributed to Plumbagin. Recently, a new bi-aphthaquinone—chitranone has been reported from *P. zeylanica* (Akella *et al.*, 1976).





Bioflavonoids

Flavonoids are the most widely distributed compounds in the plant kingdom. They are known to possess a number of useful pharmacological actions and hence it is quite appropriate to devote some time in reviewing their pharmacological properties.

A biological function of this group of compounds in man and animals was first suggested by Szent-Gyorgyl in 1936. Flavonoids were found effective in the maintenance and restoration of capillary permeability (Vit.P). However, definite conclusions on the relationship of flavonoids with capillary permeability could not be probably due to lack of satisfactory and reliable experimental models. The term Vit. P. was ultimately replaced by bioflavonoids.

There appears to be a revival of interest in bioflavonoids in recent years and their biochemical, physiological, pharmacological and therapeutic effects are being followed with great interest once again.

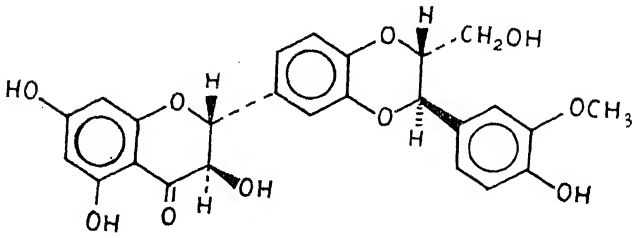
Some interesting bioflavonoids having the pharmacological actions can be considered under the following headings:

A. *Rutin,* known popularly as Vit. P. factor for decrease in capillary permeability.
Plant sources: a) *Ruta graveolens*
b) *Fagopyrum esculentum*
c) *Eucalyptus macroryncha*
d) *Hydrangea paniculata*

B. Hesperidin (Hesperitin-7-O-rutinoside)
Plant source: *Citrus sinensis*

Silybum marianum

Silymarin group comprises mainly of three isomers: Silybin (formerly also called Silymarin), Silydianin and Silychristin. Flavonolignans, almost certainly produced in the plant by a radical coupling of flavonoid and coniferyl alcohol (Hansel *et al.*, 1972).



Flavonoids are known to inhibit a number of enzymes like histidine decarboxylate, xanthine oxidase, etc. Inhibition of histidine decarboxylate forms the rationale for the clinical use of Eriodictyol in the treatment of Meniere's disease.

Recently, hydroxylethylrutinoside (HR) has been effective in the alleviation of the symptoms caused by chronic venous insufficiency of lower limbs, varicosis of pregnancy and other venous diseases.

It is thus apparent that flavonoids which were at one time considered to be of doubtful clinical usefulness and were almost condemned, have now found a definite place in today's drug armament. These compounds hold promise and if properly investigated, may provide some more clinically useful drugs which may ultimately be used as anti-inflammatory, gastric anti-ulcer, antidiabetic or for the prevention of sugar cataracts in future.

Hibiscus rosa-sinensis

Chemical constituents (Baumgarth, 1980; and Sankara Subramaniam *et al.*, 1972)
Taraxeryl acetate, -sitosterol

Flavonols

Quercetin-3-glucoside
Quercetin-3, 7-diglucoside

Anthocyanins

Cyanidin-3, 5-diglucoside
Cyanidin-3-sophoroside-5-glucoside

Antifertility Effects of the Plant (Flowers): Flower extract has shown 80 per cent antifertility in albino rats (Kholkute *et al.*, 1972). The Flower extract had a significant oral post-coital activity in female rats. The benzene and alcoholic extracts had antifertility activity of 83.7 and 50 per cent respectively (Kholkute *et al.*, 1976).

The oral administration of benzene extract of flowers (250 mg/kg daily for 30,45 and 60 days) to rats affected spermatogenesis. The endocrine function of the testes as studied by weight changes, histological and biochemical estimations (Kholkute *et al.*, 1977) was also affected.

It was my endeavour to give as wide coverage as possible, but it was difficult to choose from the material available. Some of the important plants of *Kurchi*,, *Artemesia, Mitragyana, Garlic* and *Pipers* have been left out because of lack of time and space.

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Ecdysteroids & Chemical Ecology

ECDYSTEROIDS OF FLOWERING PLANTS (ANGIOSPERMAE)

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The unusual fact that the moulting hormones of insects—ecdysones—are produced in considerable amount by some plants puts forward a number of interesting problems. There arise some questions: why do plants need moulting hormones, what is their role in the ecological interrelation between plants and insects and is it possible to employ ecdysonelike substances for practical purposes?

In the laboratory led by the author, the phytoecdysteroids of the flowering plants: *Ajuga* (Labiatae), *Silene* (Caryophyllaceae), *Serratula* and *Rhaponticum* (Composite) were investigated.

The main component of the investigated plants, phytoecdysteroids as well as in insects is ecdysterone. Besides it, eleven new ecdysteroids have been detected. Among them are: heptahydroxy and octahydroxy derivatives of 5 cholest-7-en-6-one—integristerone A and integristerone B, 2-deoxyecdysteroids—silenosterone and premixisterone, and phytoecdysteroids linked with D-galactose.

According to the author, the physiological function of the ecdysteroids in plants is connected with their influence on the protein exchange. Insects, not being able to synthesize steroids, used the anabolic properties of the ecdysonelike compounds for hormone regulation of the moulting process.

Keywords: Moulting Hormones; Ecdysteroids; Ecdysones; Chemical Ecology.

INTRODUCTION

THE moulting hormones of insects—ecdysones—are often considered as exotic substances. Nakanishi and coworkers in 1966 isolated four related compounds—ponasterones A, B, C and D while investigating the components of leaf extracts of *Podocarpus nakaii*—the plant popular in Oriental medicine. To the author's great surprise it turned out that ponasterone A had a structure close to that of α-ecdysone (I)—the moulting hormone of silkworm *Bombyx mori*. Bioassays confirmed the affinity of their action. Practically at the same time Australian chemists Galbraith and Horn (1966) isolated the authentic moulting hormone—ecdysterone (II) from *Podocarpus elata*. The number of ecdysonelike compounds found in plants started to increase rapidly. In addition to zooecdysones, the notion of phytoecdysones came into being.

The very first investigations showed that ecdysones were rather widespread in the plant kingdom. Thus, out of 1056 species (738 genus, 186 families) of Japan's

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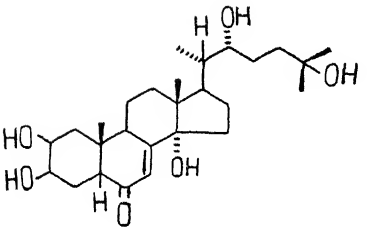
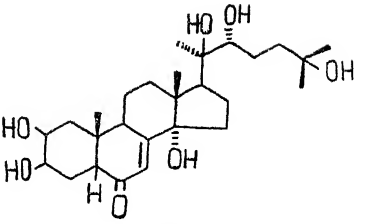


I.



II.

flora investigated, the extracts of plants belonging to 77 families possessed the activity of moulting hormones (Imai *et al.*, 1969).

Apparently, these data call for certain amendments. To establish the presence of phytoecdysones, one mainly employs biological tests based on the ability of ecydsonelike substances to cause the sclerotization (hardening and darkening) of the larva's cuticle during hibernation. More often than not the test on blue meat fly *Calliphora erythrocephala* is employed (Karlson & Bode, 1969). However, the screening of plants on the presence of ecdysones is unreliable when bioassays alone are employed. Bioassays can be employed, if at all, only for a preliminary and purely qualitative estimation of plant objects. Concerning new objects according to the traditions in the chemistry of plant substances of a positive estimation can be only such data which have been confirmed by the isolation and physico-chemical characteristics of individual compounds.

This condition considered, phytoecdysones have so far been found in approximately 90 species of plants belonging to 41 genera and 20 families (Gorovits *et al.*, 1974) (Table I).

Apparently, it is a bit too early to discuss the question about the existence of a correlation between taxonomic hierarchy and spreading of phytoecdysones in plants. It can be asserted with certainty that ecdysonelike compounds have been detected in the main parts of higher plants—Polypodiophyta, Pinophyta, Magnoliphyta. Compounds with moulting activity have not been discovered in higher fungi and weeds, but they are still poorly studied, though.

Lest the concrete compound α-ecdysone (I) should be confused with related substances, it has been proposed lately to use the term ecdysteroids (zooecdysteroids and phytoecdysteroids, respectively) instead of the name ecdysones (Scheller & Karlson, 1977). It sounds like other collective names, such as corticosteroids, cardiosteroids, etc.; and yet, 'ecdysteroids' is not a notion adequate to the moulting hormone. Of specific importance in this case is not the biological action but the affinity in the chemical structure. Such a compound may possess no moulting activity.

TABLE I

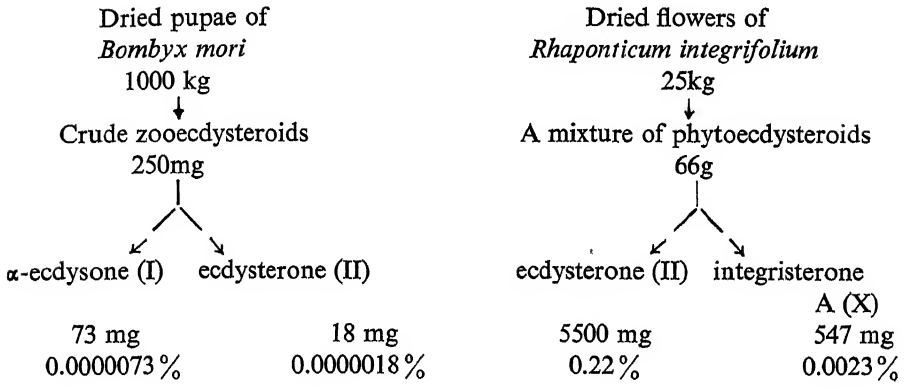
Ecdysteroids of higher plants

Family	Genus	Family	Genus
POLYPODIOPHYTA		*MAGNOLYPHYTA (ANGIOSPERMAE)*	
Osmundaceae	*Osmunda*	Commelinaceae	*Cyanotis*
Gleicheniaceae	*Gleichenia*	Liliaceae	*Paris*
Polypodiaceae	*Criosinus*		*Trillium*
	Cheilanthes	Moraceae	*Morus*
	Cyclosorus	Amaranthaceae	*Achyranthes*
	Lemmaphyllum		*Bosea*
	Neocheiropteris		*Cyathula*
	Pleopeltis		*Gomphrena*
	Polypodium		
	Pteris	Aisoaceae	*Trianthema*
			Sesuvium
Aspidiaceae	*Athyrium*		
	Dryopteris	Caryophyllaceae	*Lychnis*
	Matteuccia		*Silene*
	Onoclea	Ranunculaceae	*Helleborus*
	Thelypteris		
		Stachyuraceae	*Stachyurus*
Blechnaceae	*Blechnum*		
	Strutiopteris	Convolvulaceae	*Ipomoea*
	Woodwardia		
		Verbenaceae	*Vitex*
Pteridiaceae	*Pteridium*		
		Labiatae	*Ajuga*
PINOPHYTA (GYMNOSPERMAE)			
		Compositae	*Rhaponticum*
Podocarpaceae	*Dacridium*		*Serratula*
	Podocarpus		
Taxaceae	*Taxus*		

The advantage of plant raw materials as a source of ecdysteroids in comparison with animal organisms is of no doubt.

Firstly, the quantitative content of zooecdysteroids is, as a rule, insignificant—from hundredth fractions of a mg up to several mg per 1 kg of living weight. Thus, in the first experiments only 25 mg of α-ecdysone (I) were isolated from 500kg of silkworm pupae (Butenandt & Karlson, 1954). Then the technique was improved—250 mg of unpurified zooecdysteroids were obtained from the ton of dry pupae (Karlson *et al.*, 1963); and in plants, the content of phytoecdysteroids may reach 1 per cent and more (Baltaev *et al.*, 1978), i.e., one can obtain up to 10 kg of phytoecdysteroids from one ton of dry plant materials.

Comparative content of ecdysteroids in insects and plants



Secondly, structurally phytoecdysteroids are more diverse than zooecdysteroids. The functions of moulting hormones in insects are fulfilled by α-ecdysone (I) and ecdysterone (II). Other compounds found in their bodies (26-hydroxyecdysone, 26-hydroxyecdysterone, 2-deoxy-α-ecdysone (XIII), 2-deoxyecdysterone (XIV), 3-dehydroecdysterone, 26-hydroxy-25-deoxyecdysterone and 24-methylecdysterone) are, probably, mere metabolytes of major hormones. Almost all these substances have also been found in plants, but besides them acetates, methyl ethers, cynnamates and compound with a lactone group in the side chain have been described among ecdysonelike compounds of plant origin. But not all phytoecdysteroids possess moulting activity.

We have been studying the phytoecdysteroids of flowering plants (Magnoliphyta = Angiospermae). Our choice is conditioned by the fact that flowering plants are the ones to compose the major part of the world's green realm. By the number of species, flowering plants exceed by far all the other groups of higher plants taken together. The work seemed to us even more important because formerly it was thought that ecdysonelike substances are mostly to be found only in fern (Polypodiophyta) (Imai *et al.*, 1969).

Out of all the objects studied by us more or less full information was available only about the phytoecdysteroids of plants of *Ajuga* genus among Japan's flora. The presence of a 5- or 6-membered lactone ring in the side chain was typical for the compounds isolated from these types of plants. They compose the group of phytoecdysteroids including 29 carbon atoms and are typical of the plant world.

We have investigated overground and underground parts of the plant *Ajuga turkestanica* growing in Middle Asia, on the presence of ecdysteroids. In this object we have found substances typical for the *Ajuga* genus: ecdysterone (II), cyasterone (V) (Usmanov *et al.*, 1971), ajugalactone (VII) (Saatov *et al.*, 1977), ajugasterone B (IV) (Usmanov *et al.*, 1977). 22-Acetylcyasterone (VI) isolated from the plant's leaves in considerable amounts is a new member among the ecdysteroids of the C-29 row (Usmanov *et al.*, 1978) (Table II).

Another previously unknown compound—turkesterone (III)—accumulates mainly in the plant's roots (Usmanov *et al.*, 1973, 1975). The side chain of the new

TABLE II

Ecdysteroids containing flowering plants

Family, genus, species	Organ*	Ecdysteroids	Percentage content	References
LILIACEAE				
Paris quadrifolia L.	p	Ecdysterone	0,020	Novoselskaya *et al.* (1981)
		Polypodine B	0,010	
CARYOPHYLLACEAE				
Silene brachuica	f,l	Ecdysterone	0,030	
Boiss.	r	„	0,094	
	f,l	Integristerone A	0,020	
	r	„	0,045	
	f,l	Polypodine B	0,002	
	„	Viticosterone E	0,0012	
	r	Sileneoside A**	0,020	
	„	Silenoside B**	0,0045	
	„	Sileneoside C**	0,0032	
S. latifolia (Mill.)	f,l	Ecdysterone	TLC	
Rendle *et* Britt.	„	2-deoxyecdystereone	TLC	
	„	2-deoxy-α-ecdysone	TLC	
S. longioalycina	„	Ecdysterone	TLC	
Kom.				
S. praemixta M. Pop.	„	„	0,650	Saatov *et al.* (1979*a*)
	„	2-deoxy-α-ecdysone	0,120	Saatov *et al.* (1979*b*)
	„	2-deoxyecdysterone	0,082	
	„	Silenosterone**	0,003	
	„	Premixisterone**	0,002	
S. wallichiana	„	Ecdysterone	TLC	
Klatzsch.				
LABIATAE				
Ajuga chia Schreb.	p	Ecdysterone	0,010	
A. turkestanica Regel.	l	„	0,020	Usmanov *et al.* (1971)
	r	„	0,045	Usmanov *et al.* (1973)
	l	Cyasterone	0,025	
	r	„	0,010	Usmanov *et al.* (1975)
	l	Ajugalactone	0,001	Usmanov *et al.* (1977)
	r	„	0,001	Usmanov *et al.* (1978)
	l	Ajugasterone B	0,002	
	r	„	0,003	Saatov *et al.* (1977)
	l	22-acetylcyasterone**	0,050	
	r	Turkesterone**	0,052	
COMPOSITAE				
Rhaponticum carthamoides	p	Ecdysterone	0,276	Abubakirov (1975)
(Willd.) Iljin	l	„	0,568	
ssp. orientale (Serg.)	r	„	0,142	Yakubova *et al.* (1978)
Soskov.	„	Integristerone A	0,010	
	„	Integristerone B	0,0002	
	„	24(28)-Dehydromakisterone A	TLC	

Table II contd. on p. 127

1	2	3	4	5
Rh. integrifolium	p	Ecdysterone	0,230	Baltaev *et al.* (1978*a*)
C. Winkl.	„	Integristerone A**	0,013	
	„	Integristerone B**	0,0003	Baltaev *et al.* (1978*b*)
	„	24(28)-Dehydromakisterone A**	0,0002	Baltaev *et al.* (1974) Baltaev *et al.* (1977)
Rh. luratum C. Winkl. ex Iljin	p	Ecdysterone	TLC	
Rh. nanum Lipsky	„	„	0,007	
		Integristerone A	0,0025	
Serratula algida Iljin	f	Ecdysterone	TLC	
S. centauroides L.	l	„	„	
	„	Viticosterone E	„	
S. coronata L.	l,f	Ecdysterone	0,071	
	„	α-Ecdysone	0,003	
	„	Viticosterone E	0,0008	
S. procumbens L.	„	Ecdysterone	TLC	
	„	Viticosterone E	TLC	
S. quinquefolis MB	l	Ecdysterone	TLC	
S. sogdiana Bunge	f	„	0,520	Novoselskaya *et al.* (1975)
	l	„	0,170	Zatsny *et al.* (1970)
	f	Viticosterone E	0,0014	
	l	„	0,027	Zatsny *et al.* (1973)
	f	Sogdisterone	0,003	
S. xeranthemoides	f	Ecdysterone	0,260	Kholdova *et al.* (1979)
Bieb. (=*S. erucifolia* L.)	„	Integristerone A	0,150	

* Abbreviations: p-whole plant, f-flowers, l-leaves, r-roots.

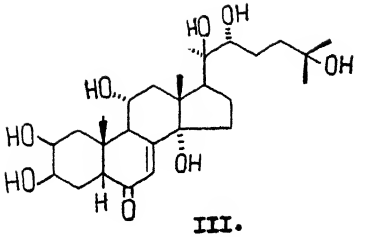
** Ecdysteroids, described for the first time by author and coworkers.

ecdysteroid does not differ from that of ecdysterone (II). The steroid part of the molecule contains an additional hydroxyl group whose position was established by spectral data. Turkesterone could have been otherwise called 11 α-hydroxyecdysterone (III).

Great importance has been attached in our investigations to the ecdysteroids of plants of the Compositae family. The plants of this family occupy the highest position in the evolution hierarchy of the plant kindgom, and the question of whether they are capable of synthesizing ecdysteroids is of paramount importance for the detection of chemotaxonomic relations.

Plants of the *Serratula* and *Rhaponticum* genera have turned out to be a good source of phytoecdysteroids. Yatzyuk and Segal' (1970) were the first to have paid attention to the presence of considerable amounts of ecdysterone in the flowers of *Serratula inermis*. We have investigated a few species of *Serratula*. In the flowers of *S. sogdiana* alongside the already known compounds—ecdysterone (II) and viticosterone E (VIII) (Zatsny *et al.*, 1971, 1973)—we have found a new substance—sogdisterone (IX) (Novoselskaya *et al.*, 1975).

No 2.



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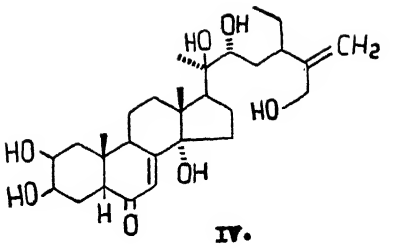

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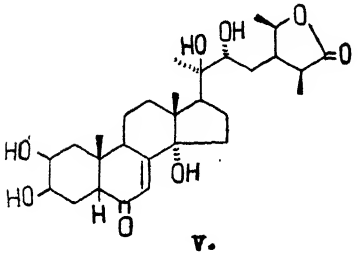

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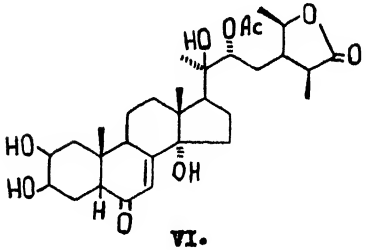

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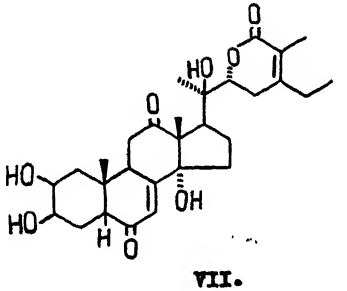

VII.

The characteristic peculiarity of the sogdisterone molecule is the presence of a hydroxyl group at C-19. A crucial point in the establishment of this fact was the absence in the PMR-spectrum of sogdisterone (IX) of the signal responding to C-19 angular methyl group. As is known, among the steroids of plant origin some cardial

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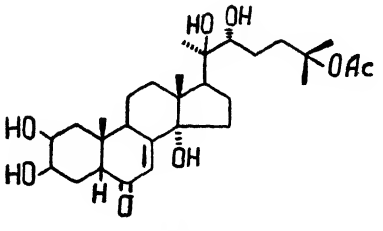

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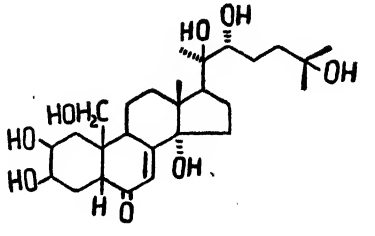

IX.

aglycones have a hydroxyl group at C-19 (strophantidol, uabagenin). There is no difference between sogdisterone and ecdysterone in the structure of the side chain. By its structure sogdisterone corresponds to 19-hydroxyecdysterone (IX).

Viticosterone E (VIII) has been earlier discovered in the leaves of *Vitex megapotamica* (Verbenaceae) (Rimpler, 1969). Its structure was established on the basis of spectral data. Single functional groups may have another configuration and that is why we found it necessary to perform partial synthesis of viticosterone E (VIII) from ecdysterone (II). The synthesis performed by two ways confirmed that viticosterone E (VIII) is 25-monoacetate of ecdysterone (Zatsny *et al.*, 1973).

Mass-spectrometric investigation of the fragmentation character of the side chain of acetates and acetonides of ecdysterone and viticosterone E made it possible to reveal certain distinguishing peculiarities, having analytical significance (Zatsny *et al.*, 1975). The data obtained were then used to establish the structure of other phytoecdysteroids.

In *S. coronata* alongside of other ecdysteroids the presence of α-ecdysone (I) was discovered, which is a rare example when the real moulting hormone is founded in plants.

A few interesting compounds have been isolated from *Rhaponticum integrifolium.* This object as well as other flowering plants contains a considerable amount of ecdysterone (II) (Baltaev *et al.*, 1974). In addition to it still other three phytoecdysteroids have been found: integristerone A (X) (Baltaev *et al.*, 1977), integristerone B (XI) (Baltaev *et al.*, 1978), and 24 (28)-dehydromakistereone A (XII) (Baltaev *et al.*, 1978).

Of specific interest are the two compounds. Both phytoecdysteroids are quite

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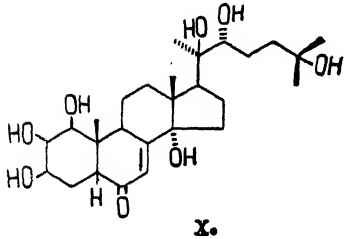
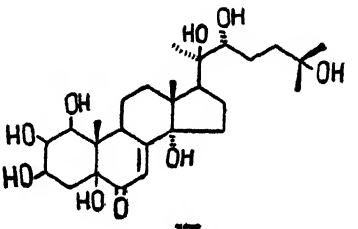
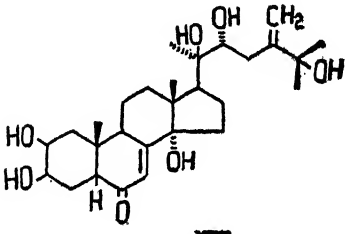


X.



XI.



XII.

hydrophylic and it is typical of them to contain a great number of hydroxyl groups in the steroid part of the molecule. Integristerone A contains only 6 and integristerone B-7 hydroxyl groups. The decisive role in the establishing of the structures of both compounds was played by the possibility to obtain isomeric and well distinguished diacetonides by hydroxyl at 1,2 and 2,3 position.

The same composition of phytoecdysteroids is observed in the legendary plant of Eastern Siberia—*Rh. carthamoides* which is otherwise known as *Leuzea carthamoides* or maral's root (Yakubova *et al.*, 1978; and Abubakirov, 1975). By its adaptogenic, tonic and stimulating action it resembles ginseng. The extract from the roots of *Leuzea* has been entered into official Pharmacopeia but it has been hitherto unknown why the plant so popular in folk medicine has a therapeutic action.

Rh. nanum growing in the alpine and sub-alpine zone of Western Tian-Shan being no bigger than a matchbox in size contains ecdysterone (II) and integristerone A (X).

Apparently, integristerone A is one of the widespread phytoecdysteroids in the plant kingdom. In addition to the above mentioned plants we have found it in *Serratula xeranthemoides* (Kholodova *et al.*, 1979) and in *Silene brachuica.*

The possibility to find ecdysteroids in the plants of the Caryophyllaceae family is quite promising. This is one of the big families among flowering plants, and its representatives are to be found in all the continents of the world including the Antarctic.

We have investigated a few species of *Silene* and found ecdysonelike substances in all of them.

The characteristic feature of the plants of this genus is the presence in them of considerable amounts of 2-deoxyecdysteroids—substances containing no hydroxyl group at C-2 of the steroid nucleus. To these, in particular, belongs the hormone 2-deoxy-α-ecdysone (XIII) found in *Bombyx mori* which is utterly important for sexual development and maturation of the testes (Ohnishi *et al.*, 1977). In the plant kingdom this compound and 2-deoxyecdysterone (XIV) close to it are typical of only *Blechnum minus*—a plant which belongs to Polypodiophyta (Chong *et al.*, 1970). The amount of 2-deoxyecdysteroids in *Silene* exceeds by tens of times that in *Blechnum.* It should be noted, by the way, that plants of the *Silene* genus displayed a negative result at bioassaying on *Chilo suppressalis,* and only the *Lychnis* genus has been positively estimated among the plants of Caryophyllaceae family (Imai *et al.*, 1969).

Two products with low polarity have been found in the flowers and leaves of *Silene praemixta* alongside of ecdysterone (II), 2-deoxy-α-ecdysone (XIII), and 2-deoxyecdysterone (XIV) (Saatov *et al.*, 1979*a*, *b*). We have named them silenosterone (XV) and permixisterone (XVI). The former contains a ketogroup at position 3 and, therefore, it can otherwise be designated as 2-deoxy-3-dehydro-α-ecdysone.

Ecdysteroids with a keto-group at C-3 have not been isolated in the native form. 3-dehydro-α-ecdysone and 3-dehydroecdysterone have merely been described as metabolytes of the major moulting hormones—α-ecdysone and ecdysterone—in homogenates of insects (Karlson *et al.*, 1972; and Karlson & Koolman, 1973). It is possible that silenosterone (XV) and premixisterone (XVI) fulfil the function of metabolytes of major phytoecdysteroids in the plant organism which is even more likely so because they have been found in comparatively small amounts.

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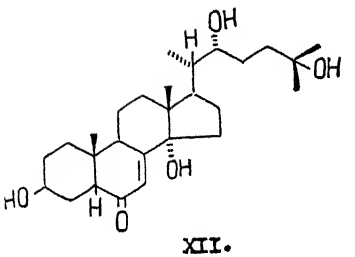

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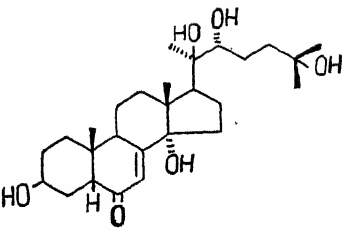
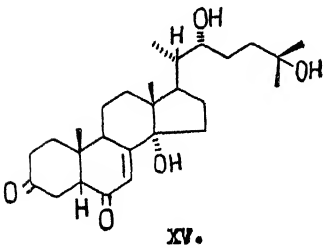


XIV.



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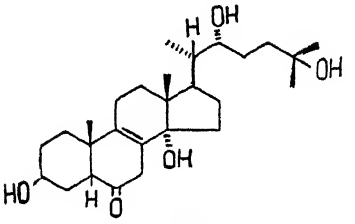

XVI.

The set of phytoecdysteroids with a number of hydroxyl groups varying from three to seven-eight has enabled us to investigate more thoroughly the fragmentation character of related substances under the influence of an electron blow (Rashkes & Abubakirov, 1980). The analysis of mass-spectra of natural ecdysteroids and their acetates has revealed certain general regularities which make it easier to establish the structure of new compounds.

The composition of ecdysteroids of *Silene brachuica* is somewhat unusual. Zoo- and phytoecdysteroids do not usually form glycosides under natural conditions. It is only known that there is but one glycoside-like compound—ponasteroside A-3 β-D-glucoside of ponasterone A—isolated from *Pteridium aquilinum* (Hikino *et al.*, 1969). That is why it seemed unexpected when addition to ecdystereone (II), viticosterone E (VIII), integristerone A(X) and polypodine B (XX) three compounds of glycoside nature were isolated from the roots of *S. brachuica* and which were called sileneosides A, B and C. Silienoide A (XVII) has the structure of ecdysterone-22-α-D-galactoside, and sileneoside B (XVIII)—that of integristerone A-22-α-D-galactoside. Of specific interest is the α-configuration of the glycoside centre formed by D-galactose which runs counter to Klyne's rule. The position of the sugar residue at hydroxyl C-22 is also unusual. An overwhleming majority of steroid glycosides are bonded with sugars *via* the hydroxy at C-3. Apparently, sileneotide C (XIX) has the structure of ecdysterone-3,22-bis-α-D-galactopyranoside. The presence of glycoside compounds of ecdysteroids in plants testifies to their active role in metabolic processes.

At present there has been established the chemical structure of approximately 60 ecdysteroids produced by the plant organism, including those described for the

first time by us. One should suppose it is far from the limit. In fact, the investigation of the plant kingdom aimed at establishing the presence of phytoecdysteroids is just beginning, and upon more thorough investigation they might be discovered in most unexpected objects.

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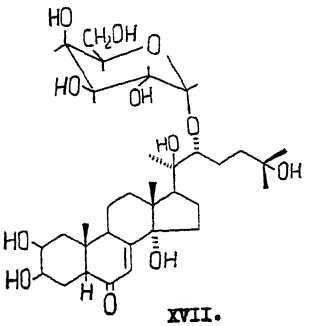
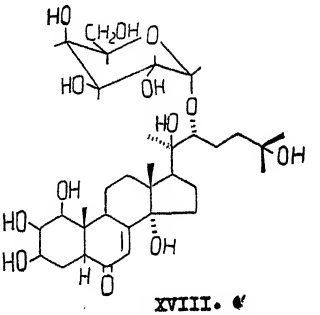
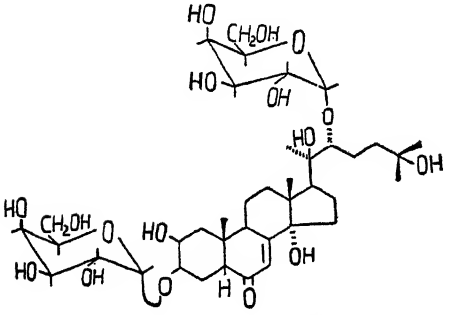


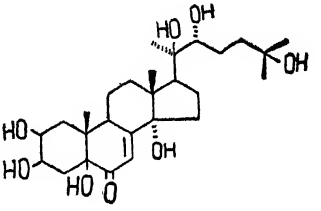
XVII.



XVIII.



XIX.



XX.

The data presented in Table II pertaining to the content of separate substances should be regarded as tentative. Depending on the place of growing and development phase of plants, the total amount of phytoecdysteroids and the correlation of certain components might vary.

What do plants need moulting hormones for?

Science is unlikely to answer this question soon, as well as the question of what for plants need alkaloids, isoprenoids, antibiotics, coumarines, tannins and other organic compounds with a comparatively small molecular weight. They used to be wrongly called secondary metabolytes and thus not so much their significance was emphasized but the dependence of their origin on the primary substances (proteins, nucleic acids, lipids, polysacharides). In the past such compounds were often identified to the final products of metabolism regarding them as waste-products.

As for ecdysteroids, of specific interest is their high physiological activity in experiments *in vivo* and *in vitro* performed in most diverse directions. Of special

attraction is the idea of the beneficial role of phytoecdysteroids in the growth and developments, i.e., a function, similar to their role in the life of Arthropoda. In this respect of utter significance is the ability of ecdysteroids to influence positively on the nitrogenous exchange. In other words, they stimulate the synthesis of proteins in the plant organism and activate the mitosis of cells. It is not accidental that phytoecdysteroids get accumulated mainly in the reproductive organs of plants—in the flowers and fruits. For example, in the flowers of *Serratula inermis* there have been discovered 2 per cent of ecdysterone (II) (Yatzyuk & Segal', 1970).

The hypothesis of the protective function of phytoecdysteroids is still more vulnerable. Artificial excess introduction of moulting hormones causes serious disturbances of the metamoprhosis mechanism which makes it possible to set them equal to insecticides. However, it should be borne in mind that ecdysteroids are not toxic and at immediate contact with insects are relatively harmless. To make their insecticide action perceptible they must come into the organism in large quantities. Larvae of the silkworm consume enormous amounts of the leaves of mulberry tree, containing ecdysterone and inocosterone, without being badly affected and rather with some use gained. In the field one can often see a plant rich in ecdysteroids and at the same time infesting in insects. In any case, ecdysonelike substances do not act as repellants.

In experiments with labelled compounds on the plants of *Podocarpus elata* (Joly *et al.*, 1969), *P. macrophyllus* (Hikino *et al.*, 1970), *Polypodium vulgare* (Souza *et al.*, 1970), it has been shown that the immediate precursor of ecdysteroids in plants is cholesterine. In the process of biosynthesis no ripping off and subsequent addition of the side chain occurs; cholesterine labelled by ^{14}C- along the C-4 of the steroid skeleton as well as an analogous compound labelled with respect to carbon C-26 of the side chain equally transform into ecdysterone (II). The biosynthesis mechanism of ecdysteroids in plants and insects, at least at its final stage, is close or identical—cholesterine labelled by ^{14}C-4 gets transformed by the larvae of *Bombyx mori* into a mixture of α-ecdysone and ecdysterone (Galbraith *et al.*, 1970).

The ability for steroid biosynthesis appeared at the very early stages of life on Earth. Sterines were produced in considerable amounts by blue-green weeds, whose age is nearly 2.5–3 billion years. Blue-green weeds emerged in the pre-Cambrian period and were the oldest autotrophic organisms capable of assimilating carbon dioxide and of discharging oxygen quite independently. This is achieved with the help of sunrays, and hence we can consider steroid compounds to be one of the first products of photosynthetic activity.

Insect appeared much later. This occurred at the end of the Paleosoic era approximately 250 million years ago. A long period of time diverts us from that ancient era. But the then existing butterflies, cockroaches and dragon-flies differed very little from the insects living now. Therefore, even then they needed ecdysteroids for their normal vital activity.

Doubtless, insects have borrowed the physiological mechanism of moulting from sea organisms. Insects are not the only class of Arthropoda which need moulting hormones for their growth and development. Ecdysterone, 2-deoxyecdysterone and related compounds have been found in the chitin covers of crawfish and crabs. Apparently, the biological process of moulting in different classes of Arthropoda regulated

by ecdysones has a lot in common, if not identical. The infection of the universal moulting hormone—ecdysterone—causes a premature moulting in crabs.

It will not be surprising if further investigations confirm the fact that the biosynthesis of zooecdysteroids in Arthropoda living in water gets realized by virtue of cholesterine. If this is true, then it means that the ancestors of contemporary insects which had left water for land borrowed the ready mechanism, which, of course, had been perfected and adapted to the existence in another environment in the course of the evolution development. This concerns not only moulting and pupation. Zooecdysteroids in insect organisms participate in the endocrine regulation of many other processes. They are indispensable for the normal functioning of the sexual apparatus and especially so, of the testes. They also influence the central nervous system, accelerate the regeneration of certain organs and ensure the alternation and completeness of biorhythms, a diapause, in particular.

It is not enough to borrow the mechanism of hormonal regulation, it is no less important that a material bearer of this function should be available. To understand the ecological connections better it would be appropriate to remember that an insect's organism cannot synthesize steroids from simple alycyclic compounds and is dependent on their arriving with the food. This is an important condition in the hypothesis advanced by us.

The chief requirements to plant food in insects and Vertebrates seem to be the same—food must contain proteins, carbohydrates, certain vitamins and some mineral salts. Together with the products providing its normal existence some other substances go into the organism of animals from plants, the first among which are such low molecular compounds as alkaloids, terpenes, organic acids, various glycosides, etc. Actually any plant possesses sufficient calorific value, but the qualitative composition of secondary metabolytes in different plants is not the same. That is why it is doubtful that the products of secondary metabolism should have any nutrient value. An animal's organism can dispose of them as it finds fit: to eliminate them through the discharge system or to utilize them for some other purposes having no connection with the permanent regeneration of primary substances.

To synthesize moulting hormones, insects have made use of cholesterine and, presumably other sterines close in structure—β-sitosterine, stigmasterine, campesterine, etc. The enzyme systems in an insect's organism are adapted *in vivo* to shorten by means of dealkylation along C-24 phytosterines with "spare" carbons and transform them into cholesterine.

Of course, we should not rule out the direct way of immediate utilization of phytoecdysteroids. By the time Arthropoda had conquered the land, the latter-abounded in fern and Gymnospermae. Could they have lent the first insects their moulting hormones?

The profuse growing of flowering plants during the early chalk period strengthened mutual adaptation, and at the same time dependence on each other of the two mighty realms of life. Being fascinated by the colouring and fragrance of flowers insects were overfond of visiting the flowers of plants and the latter spread quicker, thanks to cross-pollination. First, those branches of plants and insects received the impetus for development which were the most adapted ones for mutual existence. Their mutual adaptation was moving not only in the direction of adaptability of

morphological and anatomical properties, but in the direction of acceptability and desirability of separate metabolism products participating in the symbiosis of organisms; symbiosis benefited by the fact that the products of one partner's vital activity encouraged the growing and development of the other. Sterines including ecdysteroids might have appeared to be such mutually useful and acceptable substances.

In the process of natural selection—the main moving force of the evolution—the mutant capable of producing a metabolyte with respect to survival and gradually ousted the parent species from nature. Not all of the representatives of the green realm of antiquity are growing now—whole groups of plants have disappeared finding themselves in an evolutional deadlock.

The correctness of the hypothesis about immediate utilization of phytoecdysteroids can be illustrated by the following example (Fraenkel, 1959):

As is known, insects subsist on one or several closely related plants (monophages), a large group of plants belonging to one family (ligophages) or a still larger group of plants including several close families (polyphages). Practically, there are no insects which would subsist on all plants.

The ordinary silkworm *Bombyx mori* is a monophage. It feeds exclusively on the leaves of *Morus alba* belonging to the Moraceae family. The same family includes *Maclura aurantiaca* and *Broussonetia kazenoki,* used as living hedges and different species of *Cudrania triloba, C. javanensis* and *Ficus carica* with its edible fruit. An attempt has been undertaken at feeding silkworms with the leaves of the above plants. The larvae readily ate them, grew for sometime but sooner or later died. At any rate, they never became mature enough to pupate. However, the attempts to feed the silkworm larvae with leaves of *Urtica procera* and *Ulmus parvifolis*—plants belonging to the Urticaceae and Ulmaceae families close to the Moraceae family.

Why did the larvae die? Probably, because ecdysteroids are produced only in *Morus alba!*

The sterine composition of the above plants has not been quite thoroughly investigated so far. However, it is of no doubt that these plants contain cholesterine or, at least, β-sitosterine and stigmasterine. Sterine are the inevitable components of all plant cells. Evidently, the larvae of the silkworm do not find it sufficient to be able to transform other steroid compounds into α-ecdysone and ecdysterone. For their normal development they must get some moulting hormones with the food. The want of phytoecdysteroids increases at the stage of pupation. Probably that is why it is impossible to stop adding some amount of mulberry leaves in silkworm-growing industries in which there is a trend to use artificially composed food?

The character of netrophic relations i.e., the ones realized beyond the nutrition chain ("what serves food to whom?") between plants and insects may be more complex than given in the above example. We make a mistake, or, in any case, regard the problem in its narrow aspect when we proceed from the position of the plant itself in trying to predict the role of separate metabolytes. Acknowledging the regularity of direct connection we should not reject the existence of some back connection from insects to plants. Evolution had to deal with utterly complicated interrelation between animals and plants, both branches of life developing in dialectic unity. The specific reason connections are deeply concealed and are hard to reveal

at superficial observation. The investigation of the role of different classes of natural substances becomes justified only at the level of a population or biocenosis, i.e., a community of organisms. No matter what branch of knowledge we are developing from the molecular level to the level of the whole oganism we should regard Nature as a whole, in all its diversity.

To the three chief ecological types of adaptation—structural, physiological and behavioural—we can add still another important type—biochemical.

In conclusion, returning to the question: "Why do plants need moulting hormones?" one becomes convinced that it should have been put otherwise: "Due to what reasons did insects begin to use steroid compounds (to be more precise—ecdysteroids) inherent in the plant kingdom, as moulting hormones?"

What use can people derive from moulting hormones?

For practical purposes, the detection of plants containing considerable amount of ecdysteroids can be used in various directions:

— chemically individual moulting hormones make it possible to make out more thoroughly the mechanism of hormonal regulation of the vital functions of insects;

— by using the real raw materials foundation it will be possible to propose biological insecticides harmless for the environment;

— it is quite fascinating to employ moulting hormones in their direct designation to actively influence the silkworm metamorphosis and to increase its productiveness;

— probably, new preparation will be obtained with anabolic action to be utilized in medicine and husbandry.

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